A virosomal respiratory syncytial virus vaccine candidate with a Toll-like receptor ligand as built-in adjuvant

Kamphuis, Tobias

The research described in this thesis was conducted at the Department of Medical Microbiology, Molecular Virology Section of the University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

The work described in this thesis was financially supported by the Top Institute Pharma, Project T4-214-1 with support from Mymetics BV Leiden, The Netherlands and the University Medical Center Groningen, The Netherlands

The printing of this thesis was financially supported by:
Mymetics BV Leiden, The Netherlands
University Medical Center Groningen, Groningen, The Netherlands
Graduate School of Medical Sciences, Groningen, The Netherlands
University of Groningen, Groningen, The Netherlands

ISBN printed version: 978-90-367-5846-8
ISBN digital version: 978-90-367-5847-5

Production: Wöhrmann Print Service

RIJKSUNIVERSITEIT GRONINGEN

A virosomal respiratory syncytial virus vaccine candidate with a Toll-like receptor ligand as built-in adjuvant

Preclinical evaluation of immunogenicity, efficacy and safety

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
woensdag 7 november 2012
om 11.00 uur

door

Tobias Kamphuis

geboren op 28 november 1982
te Almelo

Promotor:	Prof. dr. J.C. Wilschut
Copromotores:	Dr. A. de Haan Dr. A.J.H. Stegmann
Beoordelingscommissie:	Prof. dr. W. van Eden Prof. dr. R.A. Karron Prof. dr. H.J. Verkade

Voor Papa en Mama

Paranimfen: Natalija van Braeckel Budimir
Paul Hendrikx

Table of contents

Chapter 1

Introduction and scope of the thesis

Tobias Kamphuis[1], Jan Wilschut[1], Aalzen de Haan[1]

1) Department of Medical Microbiology, Molecular Virology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Introduction contents

Respiratory syncytial virus: burden of disease

Respiratory syncytial virus (RSV) is the single most important cause of viral bronchiolitis in infants and young children and the leading cause of infant hospitalization in the world.[1] It has been estimated that in 2005, 33.8 million children suffered from RSV-associated acute lower respiratory infection (ALRI). Out of these, at least 3.4 million children developed severe ALRI, requiring hospitalization. In 2005, an estimated 66,000-199,000 children died of RSV-associated ALRI, 99% of whom in developing countries.[2] In the USA, RSV causes 85,000-144,000 hospitalizations of children per year and accounts for 20-25% of pneumonia cases and up to 70% of bronchiolitis cases recorded in hospitals.[1-4] Nearly all children have been infected with RSV by the age of 2 years.[5] Infection at young age, however, does not provide life-long protection against RSV and reinfections occur later in life. In healthy adults, RSV infection manifests itself with common-cold-like symptoms.[6]

The elderly and immunocompromised people are also at high risk of developing RSV-associated disease.[7,8] In the USA, approximately 180,000 people over 64 years of age are hospitalized with an RSV infection and RSV is estimated to be responsible for the death of 14,000 elderly in the USA per year. Hospitalization due to RSV infections also has a significant economic impact with annual costs exceeding $1 billion in the USA alone.[7]

The total worldwide burden of RSV disease amounts up to 64 million infections and 160,000 deaths per year. This, together with the significant economic burden, makes RSV one of the most important remaining vaccine targets.[9] There is, unfortunately, no such RSV vaccine on the market today.

Biology of RSV

Genes, proteins and function

RSV, together with for instance Measles, Mumps and Sendai virus, belongs to the family *Paramyxoviridae*, which consists of enveloped viruses with a negative-sense, single-stranded RNA genome. RSV belongs to the subfamily Pneumovirinae and, together with Para-Influenza Virus (PIV), it constitutes the genus Pneumovirus There are two main serotypes of RSV: RSV A and RSV B.[10]

The RSV genome is 15,222 base pairs long and codes for 11 viral proteins (Figure 1.1). The genome is transcribed from the 3' end to the 5' end with only a fraction of the polymerase moving on to the next gene, resulting in a gradient of transcription reflecting the required relative abundance of the encoded proteins.[10]

The non-structural NS1 and NS2 proteins are located at the 3' end of the genome and are therefore the most abundantly transcribed RSV proteins. RSV NS proteins play an important

role in immune evasion mechanisms: they inhibit virus-induced type-I interferon responses of the host through inhibition of RIG-I, IRF-3 and proteosomal degradation of Stat2.[11-13]

The nucleocapsid proteins nucleoprotein (N) and phosphoprotein (P) are encoded downstream of the NS proteins and are essential for transcriptional activity.[10] The matrix (M) protein is located underneath the lipid bilayer of the assembled virus particle and is important in inhibition of the polymerase prior to virus assembly.[10] The small hydrophobic protein (SH) forms an ion channel that spans the viral membrane. Its function in RSV infection or replication is still unknown.[10,14]

RSV attachment and fusion glycoproteins G and F are encoded downstream of SH. These RSV proteins induce virus-neutralizing antibodies upon infection of the host which makes them excellent RSV vaccine targets.[10] The F protein shows a relative conserved sequence among different strains, while sequences of G exhibit more variation. Differences in G protein sequences are also the main determinants of the RSV A/B serotype division.[10] The G protein mediates binding of the virus to the host cell membrane through binding to cell-surface proteoglycans. Despite the role of G protein in attachment of RSV to host cells, it is not absolutely required for RSV infection of *in vitro* cultured cells.[15] Virus lacking the G protein grows to high titers in culture but is attenuated in its replicative capacity upon infection of cells lining the respiratory tract.[16] Furthermore, a proportion of the G protein that is synthesized upon infection is produced in soluble form (sG). This sG has been shown to affect a proper immune response to RSV infection or vaccination.[17] sG has also been shown to inhibit type-I IFN induction through Toll-Like Receptors (TLRs), such as TLR3 and TLR4,[18] underlining the role of this protein in immune evasion.

The F protein is absolutely required for RSV infection: it mediates fusion of the viral membrane with the host cell membrane, facilitating deposition of the RSV genome into the host cell.[10] A recent study has suggested nucleolin to function as the cellular receptor for RSV-F.[19] F is produced as a 67 kDa precursor (F_0) which undergoes proteolytic cleavage into F_1 and F_2. These two subunits form the mature pre-fusion F which, in the membrane, forms a homotrimer.[20] During fusion, this homotrimer undergoes a conformational change leading to insertion of the fusion peptide in to the membrane, facilitating membrane fusion. RSV F fusion occurs at the plasma membrane and is pH-independent.[21] The precise trigger for RSV fusion, however, is still unknown. When, during infection, F is expressed on the cell surface of infected cells it also facilitates cell-cell fusion leading to the formation of syncytia.[10]

The M2 protein is encoded downstream of F and has two open reading frames resulting in the transcription of M2-1 and M2-2.[20] M2-1 functions as a transcription elongation factor.[22]and M2-2 is involved in virus transcription regulation.[23]

The long gene (L) is located at the 5' end of the genome and is therefore the least abundant RSV protein. The L gene codes for the polymerase, which facilitates replication of the viral RNA and

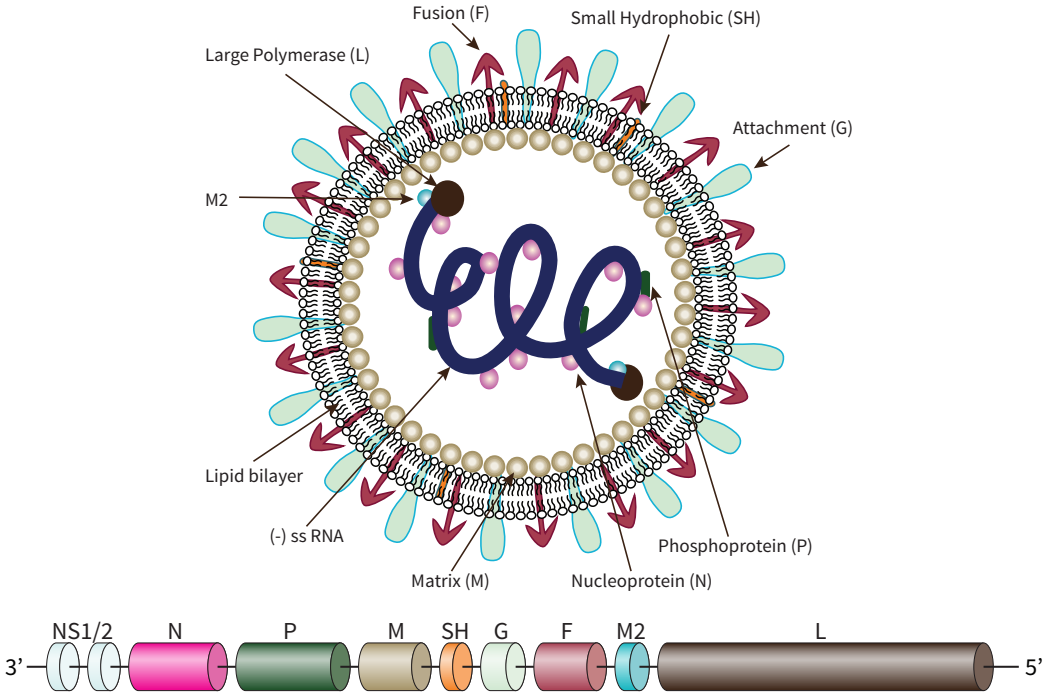


Figure 1.1. RSV virus particle and genome. Schematic representation of an RSV virus particle (above) and the RSV single stranded negative-sense genome (below)

is also implicated in capping, methylation and polyadenylation.[24]

Infection, replication and virus assembly

RSV transmission occurs though contaminated nasal secretions via large respiratory droplets by either close contact with an infected individual, or a contaminated surface. Aereosolized secretions appear to be less important in RSV transmission.[25] Upon entry in the respiratory tract, RSV infects the airway epithelium.[10] As indicated above, this involves attachment of the G protein to cell-surface proteoglycans followed by binding of the F protein to its receptor nucleolin, leading to membrane fusion which delivers the viral ribonucleoprotin (RNP) in to the target cell cytosol.[19,26] Inside the cell, the viral genome is transcribed by the polymerase complex (L, N, P, M2-1) and subsequently translated by cellular ribosomes. When viral proteins start to accumulate in the cell, the virus switches from transcription to virus genome replication.[27] The viral glycoproteins travel through the Golgi complex to the membrane. The glycoproteins, matrix protein and the viral RNP assemble at lipid rafts and new virus particles subsequently bud of from the cell membrane.[28] Another mechanism of infection is by F-mediated cell-cell fusion which effectively delivers viral RNP to neighboring uninfected cells.[29] Cell to cell spread of RSV *in vivo* is the most efficient replication mechanism for the virus leading to infection of neighboring cells while evading detection by the immune system.[30]

Pathogenesis of RSV infection

Upon entry of RSV in the respiratory mucosa, the virus replicates in the nasopharyngeal epithelium in the upper respiratory tract and will subsequently move to the lower respiratory tract after one to three days.[25,31,32] In the lower respiratory tract, RSV causes bronchitis, bronchiolitis and pneumonia.[1] RSV pathology is caused by viral, immunological and host-genetic factors.[1] RSV F-mediated fusion of infected cells with non-infected cells leads to the formation of large masses of cells with multiple nuclei, i.e. syncytia.[33] These necrotic clumps of cells induce mucus secretion in the airways leading to airway obstruction. The formation of syncytia is followed by peribronchial infiltration of lymphocytes, neutrophils, eosinophils, plasma cells and macrophages contributing to immunologically-mediated pathology.[33]

A wide range of single nucleotide polymorphisms (SNP) in host immune response genes have been correlated with disease severity.[1,34] SNP in genes of the innate immune response such as transcriptional regulator Jun, IFN-α, nitric oxide synthase and vitamin D receptor show the strongest correlation with disease severity.[35,36]

In more severe disease, the infection progresses to the lower respiratory tract, leading to pneumonia with interstitial infiltration, alveolar filling and eventually, in the worst cases, the death of the patient.[31] In most cases, however, recovery begins within several days after the start of the symptoms and symptoms disappear after 7 to 10 days.[25] The airway epithelium is fully restored in four to eight weeks after initiation of the infection.[31]

Immune response to RSV infection

The immune response against RSV infection is a double-edged sword: On the one hand, it is required for clearance of the virus; on the other hand, it also contributes to immunopathology, as described above. The innate, humoral and cellular arms of the immune response all play a vital role in protection against RSV infection.

Innate response

The first line of the host's defense against an RSV infection is the innate immune response. Innate immunity against RSV serves to prevent viral replication and spread of the virus, while, at the same time, priming the adaptive immune system for an adequate response. The innate immune system contributes to protection with its own antiviral mechanisms, such as the production of type-I IFN and activation of NK cells.[37,38] The most important role however, of innate immunity is to prime the adaptive immune system in such a way that it adapts its responses to effectively clear the virus and establish an adequate protection against reinfection.[39]

Adaptive immune responses against infections are tailored to protect the host in an efficient and pathogen specific manner. Infection with an extracellular parasite for instance, can be

eliminated by antibodies while the clearance of intracellular virus infection require cytotoxic T-cells.[40] The establishment of an immune response in one of these directions is governed by $CD4^+$ T-helper cells. These can differentiate from naïve, Th0 cells in to either Th2 cells which give rise to an immune response suited for protection against extracellular pathogens or Th1 cells will induce an immune response suited for protection against intracellular pathogens such as viruses.[40].

To establish an effective immune response against RSV, it is important that a Th1-response is induced. While some events during RSV infection give rise to such a Th1-type response, RSV has evolved mechanisms that skew immunity towards a less effective Th2-type response.[41] A number of viral proteins have direct effects on the host's cytokine and T cell response, as indicated below.

When RSV binds to host cells, the F protein is recognized by TLR4 expressed on the cell membrane which induces the production of IL-1β IL-6, IL-8 and TNF-α.[42] Mice deficient in TLR4 showed impaired natural killer (NK) cell activity, reduced IL-12 expression and reduced capability to clear the virus.[43] Polymorphisms in TLR4 have been shown to influence the severity of disease in children, indicating that TLR4-mediated detection of RSV also plays a role in protection against RSV in humans.[44] Besides the induction of the cytokines mentioned above, RSV infections also induce secretion of cytokines and collectins such as ICAM-1, IL-8 and RANTES (Regulation upon Activation Normal T cell-Expressed and Secreted) that initiate neutrophil, CD4+ and eosinophil chemotaxis.[45-47]

Replication of RSV leads to the production of double stranded RNA which activates RIG-like helicases RIG-I and MDA-5.[48] Activation of RIG-I normally leads to activation of the transcription factor IRF-3 and, consequently, to production of type-1 IFNs like IFN-β.[48] During RSV infection however, this pathway is inhibited by the RSV non-structural NS proteins.[13]

RSV G and sG proteins also influence the innate immune response as well as adaptive T cell responses, stimulating, for example, Th2-skewing of the cellular immune response.[41] RSV G contains a domain that mimics the CX3C domain of fractalkine (FKN), a chemokine known to attract neutrophils and eosinophils. Furthermore, a T cell antigen domain in RSV G (aa181-192) induces release of high amounts of the Th2 cytokines IL-4, which inhibits the differentiation to Th1 cells.[41] It further induces IgE subclass antibodies involved in hypersensitivity reactions. Other induced cytokines involve IL-5, an activator of eosinophils, IL-10 and IL-13. The inhibition of Th1 cell-produced cytokines such as IFN-γ, mediated by these cytokines diminishes viral clearance and correlates with disease severity.[49]

It is clear that RSV infection induces multiple factors that drive both Th1 and Th2 responses. Activation of TLR4 by RSV F and RIG-I by dsRNA produced during replication triggers factors

that enhance Th1-type responses suitable for effective inhibition of viral replication On the other hand, the NS proteins inhibit RIG-I signaling and sG induces Th2 cytokines that favor the formation of IgE and chemo-attraction of neutrophils and eosinophils, thus diverting the immune response a phenotype less suited for inhibition of viral replication and to the resolution of disease. The balance between these mechanisms determines to a considerable extent the severity of RSV-induced illness.

Cellular response

After establishment of an infection, the cellular branch of the adaptive immune response is important to control the infection.[49] Patients with impaired T cell immunity showed more severe disease and increased virus shedding.[50] CD8+ Cytotoxic T lymphocyte (CTL) responses are elicited against NS2, N, M, M2, F and SH proteins with responses against F, N and M2 being the most prominent.[51] CD4+ T-helper cells express a wide range of cytokines, including IL-2 and IFN-γ, contributing to the efficacy of the CTL response. Studies in BALB/c mice have shown that early expression of IFN-γ is especially important for priming of an adequate CTL response.[52]

Humoral response

CTL are instrumental in clearing virus after infection, however, they have limited effect in protecting against infection. Antibodies on the other hand are able to provide protection against reinfection. Passively acquired maternal RSV-specific antibodies protect newborns against infection during the first couple of weeks of life, but levels of these antibodies wane rapidly.[53] High levels of maternally-acquired antibodies correlate with absence of severe bronchiolitis upon RSV infection.[54] The monoclonal anti-F antibody Paluvizumab is administered to children from high-risk-groups such as preterm infants and children with congenital heart disease and protects against lower respiratory tract illness.[55] After primary infection, transduced and locally produced IgA predominantly protects the upper respiratory tract against reinfection.[56] In the lower respiratory tract, high-affinity virus-neutralizing IgG antibodies have been demonstrated to provide protection.[56] The G protein-mediated induction of high levels of IL-4 also causes B cells to undergo class-switching to produce IgE which has been associated with more severe disease and wheezing.[57,58] In general, the levels of induced antibodies wane rapidly and quickly fall below a protective threshold level. During reinfection, levels of neutralizing antibodies rise faster than during primary infection which contributes to a milder course of disease after secondary infection.[56] An RSV reinfection in adults induces a rise in antibody levels, which within 2 years recede back to levels that are not sufficient to confer protection.[59] For protection of the elderly, RSV vaccination should result in long-lasting protective levels of neutralizing antibodies. It is unlikely that an immunization strategy induces neutralizing antibodies at levels that last longer than those induced after natural infection. This would probably require vaccination to be given on a yearly basis.[59]

Treatment of RSV infection

As mentioned above, the monoclonal antibody Palivizumab is registered for prophylactic administration to children in high-risk groups.[60,61] Palivizumab is also sometimes used as a treatment to stop progression of upper respiratory tract infection to the lower respiratory tract, however the efficacy and cost-effectiveness of this treatment are debatable.[62] Apart from Palivizumab, aerolized Ribavirin is also registered for treatment of RSV in children. Its efficacy in children is controversial in children as not all the randomized controlled trials conducted to evaluate this treatment have demonstrated a significant effect.[63] The evidence for efficacy of Ribavirin to reduce RSV replication in adults is not assessed in clinical trials and merely anecdotal.[64] Currently, RSV treatment in adults consists of symptomatic relief and includes administration of fluids, supplemental oxygen and bronchodilators in the most severe cases.[65] Because of the limited efficacy of treatment available for RSV-infected patients, a vaccine that could provide protection against infection would significantly lower the burden of RSV disease.

Early vaccination strategies against RSV

Formalin-inactivated vaccine

After the success of the killed polio vaccine[66] and the recognition that RSV posed a serious threat for infants, a formalin-inactivated RSV vaccine was developed and evaluated in children.[67] A prior small-scale trial had shown increases in immunogenicity without any adverse effects. This warranted further study and four clinical trials were conducted, which ran between 1965 and 1967 in the United States.[67-70] Two of these trials were held on military bases, Fort Ord in California and Lowry airforce base in Colarado, one in a center for homeless children in Washington DC and one in children from the community around Washington DC. The FI-RSV vaccine was developed by Pfizer and consisted of a concentrated formalin-inactivated alum-absorbed RSV preparation. The Bennett RSV strain was used to generate the vaccine: it was first propagated four times on primary human embryonic kidney cells and subsequently ten times on vervet monkey kidney cells.[68] In 3 trials, children of 6 months of age and older were vaccinated. The trial in children from the Washington community also included children below 6 months of age.

Reports from all four trials indicated that vaccination with FI-RSV did induce serum antibody levels, as measured by complement-fixation techniques, with a four-fold increase in antibody levels in almost 90% of the vaccinees. The rise in antibody levels was highest in the oldest children.[69] The outcome of virus neutralization titer determination differed between trials and age groups. In the youngest children, only a small increase in virus neutralization titers was found,[68] while in older groups larger increases in neutralization titers were found. Approximately 50% of the non-vaccinated children also had high levels of neutralizing antibodies due to previous infections. Up to 72% of the vaccinated children above the age of one year showed high neutralizing antibody titers.[67] The presence of neutralizing antibodies in both the vaccinated and in the non-vaccinated group correlated with reduced virus shedding after RSV infection, but not

with the onset of pneumonia.[67] Strikingly, 69%-80% percent of vaccinated children developed pneumonia after infection compared to only 9% of the non-vaccinated children. Hospitalization rates were 4-fold higher in vaccinated children compared to those in non-vaccinated children.

In the trial which included the youngest infants, 91% of the children had RSV antibody CF titers but only 43% showed an increase in virus-neutralizing titers.[68] From the children vaccinated with FI-RSV, 65% were reported to having subsequently acquired an RSV infection: 80% of these children required hospitalization. Another group included in this study was vaccinated against parainfluenza. Of these children, 53% acquired an RSV infection but only 5% of the infected children required hospitalization. Of the 18 hospitalized children, 2 died as a result of complications following infection. Postmortem analysis of the lungs of these two children showed that they suffered from extensive bronchopneumonia. Microscopic analysis of lung samples showed the presence of peribronchial monocytic infiltration with some excess in eosinophils. At least 10^4 $TCID_{50}$ RSV could be recovered from the lungs of the deceased children.[68] At the time, knowledge about immunological mechanisms that contributed to this phenomenon was scarce, although parallels were observed between FI-RSV-mediated enhanced disease and a-typical measles cause by a killed measles vaccine formulation.[70,71]

Enhanced respiratory disease

Following the disastrous outcome of the four FI-RSV clinical trials, the emphasis in the RSV field moved to elucidating the underlying mechanisms responsible for the development of enhanced respiratory disease (ERD). The first analysis performed on the clinical trial samples showed that, although antibody titers determined by ELISA against the F and G protein were high, these antibodies had lower neutralizing capacity compared to those induced by live virus infection.[72,73] Not only did these antibodies fail to protect from infection, they possibly have also contributed to ERD by the formation of immune complexes, which fix chemotactic complement factors that attract, for instance, neutrophils.[74,75] Later studies in mice showed that the failure of the antibodies to neutralize the virus was primarily due to poor affinity maturation, which in turn was caused by the lack of adequate TLR signaling by FI-RSV.[76]

Besides the induction of poorly neutralizing antibodies, studies in mice and cotton rats have also shown that FI-RSV skews the adaptive immune response to a Th2-phenotype.[77,78] Anti-IL-4 treatment of mice at the time of FI-RSV vaccination led to decreased clinical symptoms.[79] Furthermore, it was shown that FI-RSV vaccination in cotton rats followed by infection with live virus stimulated a wide range of Th2-associated cytokines as well as several Th1-type cytokines. Addition of the TLR4 ligand monophosphoryl lipid A (MPLA) during vaccination reduced the cytokine expression levels and inhibited lung pathology in cotton rats.[78]

The exact mechanism by which FI-RSV leads to the induction of ERD after infection is unknown and most likely multifactorial. Antigen alteration by formalin,[80] the presence of cell culture

proteins[81] and the use of alum as an adjuvant[82,83] all have been shown to contribute to ERD. As successful RSV vaccine should therefore induce high levels of RSV-neutralizing antibodies and should not skew the immune response excessively a Th2-phenotype.[84]

Current RSV vaccine strategies under development

A wide range of approaches including the use of live attenuated RSV, subunit or purified viral proteins, chimeric viruses and vector-based vaccines, have been or still are under development as candidate RSV vaccination strategies. Several of these have been tested in clinical trials, but so far, none of them has met the required safety and efficacy criteria. To illustrate a number of the difficulties associated with RSV vaccine development, several of these approaches are discussed below.

Live attenuated vaccines

The most extensively evaluated approach to vaccination against RSV is the use of live attenuated virus vaccines and this is the only strategy so far that has recently been tested in seronegative children, 1-2 months of age. With attenuated RSV vaccines, however, the balance between immunogenicity and attenuation appears to be difficult to establish.[84] A number of live attenuated vaccines have been evaluated in clinical trials from *cp*RSV in 1968 to *ts*-1 and *ts*-2 in the early eighties.[85] The first live attenuated vaccine to be tested in children was *cpts* 248/995[86] and *cpts* 248/404 was the first to be evaluated in 1- to 2-month-old infants. Immunization with this vaccine caused high incidence of nasal congestion and was considered to be insufficiently attenuated for use in the youngest children.[87] To further attenuate the vaccine the SH gene was deleted and an additional mutation was introduced (rA2cpts248/404/1030/ΔSH). This virus did not lead to nasal congestion in infants and induced a measurable immune response in 44% of the vaccinees. These responses however did not correlate with protection in this study and more studies are required to show protective efficacy of this vaccine.[88]

During immunization with attenuated vaccines, low levels of virus replication occur leading to selection for viruses with a higher replicative potential, possibly reversing the attenuating mutations. Virus recovery from the children who received A2*cpts*248/404/1030/ΔSH showed that 1 of the 5 mutations were lost.[88] These revertants however did not show increased virulence compared to the vaccine strains. Clinical trials in larger populations will have to demonstrate whether the virus retains sufficient numbers of attenuating mutations and will not revert back to a pathogenic virus.

Subunit or purified protein vaccines

Purified protein vaccines are relatively straightforward to produce and are efficacious in, for instance, HepB and HPV vaccination strategies. A purified RSV F protein (PFP-2 and PFP-3) adjuvanted with alum has been evaluated in pregnant women, healthy adults over 60 years of age and children with cystic fibrosis (CF).[89-91] In the case of pregnant women, the vaccine

induced a greater than 4-fold increase in virus-neutralizing antibodies only in 10% of the vaccines.[90] Antibodies against F in newborns and breast milk from vaccinated mothers, however, were significantly increased. In elderly, this vaccine induced a greater than 4-fold increase in neutralizing antibodies in 61% of the vaccinees. In children with CF, the vaccine induced a greater than 4-fold rise in neutralizing antibodies in 67% of the vaccinees. Group sizes in these trials were insufficient to show a significant reduction in RSV infection in the vaccinated groups.[92]

Another approach using a vaccine consisting of purified F, G and M proteins induced a greater than 4-fold rise in neutralizing antibodies in 58% percent of vaccinated individuals over 65 years of age. A possible explanation for the good immunogenicity of these purified protein preparations could be that they form virus like particles. This was previously shown with Newcastle disease virus (NDV), another paramyxovirus.[93]

A G protein fragment fused to an albumin binding region (BBG2NA) produced in *E. coli* showed promising results in preclinical studies, but induced only moderate increases in virus-neutralizing antibodies in healthy adults.[94,95] Studies in rhesus macaques demonstrated the inability of this vaccine to induce protection against virus infection and also showed undesired induction of detectable eosinophils and expression of Th2-cytokine IL-13 in the lungs.[96]

Furthermore, several other vaccine approaches are currently being evaluated in preclinical and early-clinical trials.[97,98] To date, no purified protein vaccine has been tested in seronegative children. In order for a candidate vaccine to proceed to clinical trials in seronegative children, it should first be well established that these vaccines induce a robust neutralizing antibody response and skew the T-cell response away from a Th2-phenotype.[84] In order to achieve this, a non-replicating RSV vaccine could benefit from the use of an immunomodulating adjuvant.

Chimeric live virus

Other approaches for RSV vaccination include the use of chimeric viruses which have the ability to replicate but do not cause disease. These viruses express RSV genes and immune response are mounted against RSV gene products upon infection. A chimeric virus consisting of Bovine PIV3 expressing RSV F and hPIV F (MEDI-534) is so far the only chimeric vaccine approach tested in the clinic. In a preclinical evaluation in African green monkeys, this vaccine induced protection against infection with wild-type RSV, even though the levels of neutralizing antibodies induced were low.[99] In clinical tests, the vaccine proved to be well tolerated and safe. However, the capacity of the vaccine to induce neutralizing antibodies was limited in humans as well.[100,101] In a study in seronegative children between 6 and 24 months of age, the vaccine showed acceptable safety and a greater than 4-fold increase in neutralizing antibody levels was demonstrated in half of the children.[102] A phase 1/2a study evaluating protection against infection is currently underway.[102]

Vector-based vaccines

Virus replicon particles (VRP) derived from the *Alphavirus* Venezuelan Equine Encephalitis virus (VEE) expressing RSV F (VRP-RSV.F) have been evaluated in mice and cotton rats. These studies demonstrated induction of mucosal IgA, which would be beneficial in protection against RSV infection.[103]Furthermore, the VEE replicon vaccine has proven to be immunogenic and to refrain from skewing to a Th2-type immune response.[103] As yet, this approach has not been tested in a clinical setting.

Vaccine considerations

The number and diversity of vaccine approaches currently being tested in preclinical and clinical stages demonstrate the high demand for an RSV vaccine. Unfortunately, all the approaches currently being tested have revealed major drawbacks and the ideal RSV vaccine is yet to be found. The use of attenuated viruses is difficult because of the delicate balance between attenuation and immunogenicity. Both attenuated RSV and live chimeric vaccines require careful storage and transportation and have a limited shelf life. This is especially challenging in the developing world, where RSV causes the highest mortality rates. Furthermore, purified protein approaches have only shown limited immunogenicity in clinical trials and some tendency to skew towards a Th2-type response in animal models. This urges for different approaches that more specifically address these issues, as discussed below.

Virosome technology

Another vaccine approach that might prove useful to protect against RSV, involves the use of virosomes. Virosomes consist of reconstituted viral envelopes that contain the surface glycoproteins of the virus but lack the viral nucleocapsid. The first virosomes were developed in 1975 on the basis of influenza virus.[104] Influenza proteins hemaglutinin (HA) and neuraminidase (NA) were purified and inserted into unilamellar liposomes made from phosphatidycholine (PC) and phosphatidylethanolamine (PE).[104] Subsequently, proteins from HIV, EBV, Senbis virus and Rabies virus have been used in a similar approach to generate virosomes.[105]

The HA in influenza virosomes mediates receptor-mediated endocytosis similar to that induced by live influenza. Because the HA also retained its membrane fusion capacity, virosome encapsulated protein antigens have been shown to be delivered into the cytoplasm of antigen presenting cells, where proteasomal degradation of antigen occurs and antigen-derived peptides can be transported to the ER for loading on MHC class I molecules thus priming for CTL induction.[106] Influenza virosomes have also been shown to enhance immune responses towards “third party” antigens[107] leading to the use of influenza virosomes as carriers for other viral antigens such a Hepatitis A.[108,109]

In addition to using liposomes for insertion of influenza HA and NA proteins, virosomes can also be produced by solubilization of the viral membrane by the detergent octaethyleneglycol

mono(n-dodecyl)ether ($C_{12}E_8$), nucleocapsid removal and subsequent reconstitution of the solubilized viral membrane by addition of detergent-binding beads.[110] $C_{12}E_8$-mediated solubilization of membranes and removal of the detergent by beads is a laborious process. Dialysis would be a preferred method for detergent removal and viral membrane reconstitution. However, $C_{12}E_8$ has a low critical micelle concentration (cmc) making dialysis inefficient.[111] The short-chain phospholipid 1,2-dicaproyl-*sn*-glycero-3-phosphocholine (DCPC) can also solubilize virus membranes without affecting the membrane proteins. Removal of DCPC by dialysis is possible because of its relatively high cmc.[111] So far, the DCPC approach has been used only for production of influenza virosomes. RSV, however, is a similar enveloped virus and therefore solubilization and reconstitution of RSV membranes using DCPC could represent a viable approach for making a virosomal RSV vaccine. Through removal of the nucleocapsid, RSV virosomes lack the viral genome and, thus, are non-replicating virus-like particles.

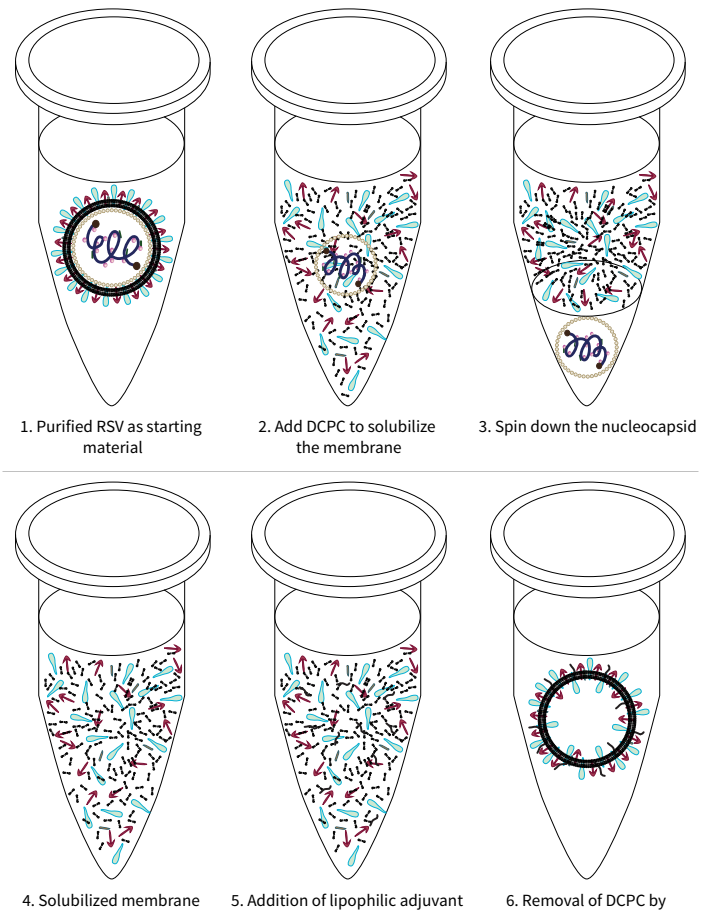


Figure 1.2. Virosome production. Schematic representation of the production of RSV virosomes with TLR ligands as adjuvants. Purified RSV is solubilized with DCPC. The nucleocapsid is removed by ultracentrifugation. The lipophilic adjuvant is added to the supernatant and the DCPC is removed by dialyses, reconstituting the viral envelopes containing the membrane glycoproteins and inseterted TLR ligand

TLR ligands as vaccine adjuvants

General, TLR ligands in vaccines

A promising approach to modulate the immune response induced by vaccination is the addition or inclusion of Toll-Like Receptor (TLR) ligands.[112] TLRs are a very conserved component of the innate immune system. They belong to the family of pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). Unique components of bacteria or viruses are recognized by TLRs expressed on the surface or in endosomes of a wide variety of cells.[113] TLRs are instrumental in directing the adaptive immune response towards a Th1 or Th2-type response.[114] Activation of TLRs and their down-stream signaling pathways through adapter molecules MyD88 and TRIF induces the production of inflammatory cytokines and type-I interferons which in turn directs the differentiation of naïve Th0 cells to Th1 or Th2-type $CD4^+$ T-helper cells. [40]

TLR ligands have (unintentionally) been a part of a wide variety of human vaccines starting with the rabies virus vaccine introduced in 1886, which contained singl-stranded RNA.[115] It took until the 1990s for TLR to be recognized as the molecules responsible for the recognition of PAMPs from pathogens and in 2005 the first vaccine with an intentionally added TLR ligand was approved for use in humans.[115] The ability of certain TLR ligands to skew the immune response to a Th1-type response and, at the same time, to stimulate processes like antibody production make them an excellent potential additional component of an RSV vaccine.[76] The production process of the reconstituted virosomes allows incorporation of lipophilic adjuvants, for instance lipophilic TLR ligands, into the membrane during the reconstitution. The association of the antigen (virosomal proteins) with the adjuvant (TLR ligand) fully mimics natural pathogens and allows instant activation of antigen-presenting cells, such as dendritic cells that take up the antigen. This leads to a more efficient response.[116] Furthermore, lipophilic TLR ligands benefit from incorporation into lipid membranes as this more closely represents their native conformation. Two lipophilic TLR ligands that are of particular interest for inclusion into a virosomal RSV vaccine are lipopeptide TLR2 ligand *N*-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-$(lysyl)_3$-lysine (Pam3CSK4) and the LPS-derived TLR4 ligand monophosphoryl lipid A (MPLA).

Pam3CSK4

Lipoproteins are found in the membrane of Gram-negative bacteria, Gram-positive bacteria and mycoplasma.[117] In the 1970s it was observed that lipoproteins stimulate B-cell growth.[118] Later it was shown that they also activate the transcription factor NFκB and cytokine production.[119,120] Synthetic analogues of the N-terminus of lipopeptide of the *E. coli* lipoproteins have been shown to be as active as the natural lipoprotein.[121,122] The synthetic lipopeptide Pam3CSK4 was shown to more effectively induce CTL when compared to other adjuvants.[123] Similar to a large number of known TLR ligands, the notion that Pam3CSK4 signaling occurs through TLR2 was only discovered after it was long known that this molecule had beneficial adjuvant effects.[124]

The lipophilic properties and immunomodulating properties of Pam3CSK4 make it a suitable candidate as an adjuvant for use in a virosomal RSV vaccine, as it could boost the formation of (neutralizing) antibodies and modulate T helper type responses.

MPLA

Another lipophilic TLR ligand possibly suited for use in a RSV virosomal vaccine is the detoxified LPS derivative MPLA. The first molecule that was recognized as a TLR ligand was lipopolysaccharide (LPS), which signals through TLR4.[125] Extreme toxicity however, prevented LPS to be useful in human vaccines. In the 1980s Edgar Ribi and colleagues modified the LPS of *Salmonella Minnesota,* leading to the discovery of a compound that lacks the toxicity of LPS but retained the its immunomodulating activity.[126] This led to the development of MPLA as an adjuvant component currently used in two licensed human vaccines.[127] MPLA has been shown to stimulate the immune response against the antigens with which it is co-administered and has an acceptable safety profile in humans.[127,128] Furthermore MPLA has been shown to suppress symptoms of RSV ERD when co-administered with FI-RSV to cotton rats,[78,129] suggesting that the immunomodulatory function of MPLA is beneficial in RSV vaccination.

Animal models for RSV vaccine evaluation

In order to elucidate RSV infection mechanism and evaluate candidate vaccines, animal models are indispensible. Unfortunately infection with human RSV infection is strictly confined to humans and none of the available animal models fully replicate all aspects of human disease.[130] Non-human primates (NHP) have been used for assessment of the level of attenuation of live-attenuated vaccines, even though they do not undergo severe RSV infection.[131] African Green Monkeys are the most RSV-permissive NHP and have also been used as a model of FI-RSV-induced ERD.[132,133] Due to costs and logistical problems associated with NHP as an animal model, this species is less suited for use in early stage preclinical vaccine evaluation.[134]

RSV was initially discovered as a chimpanzee coryza agent[135] and also infects other non-human primates such as African green monkeys.[136] The economical, ethical and emotional burden of working with chimpanzees is extremely high. Furthermore, there are no inbred strains and the experimental sample size is typically small.[134] Therefore non-human primates are not the most suitable animal model when it come to early preclinical vaccine evaluation.

Bovine RSV is very much related to human RSV and infects cattle. There are several advantages to using cattle as an RSV infection model, such as similarities in infection with humans and the possibility to perform respiratory function analysis in these animals. However, their size, associated costs and the lack of available reagents for immunological studies makes this species less suited for preclinical RSV vaccine evaluation.[130,134]

Rodent models such as the Th2-biased BALB/C mouse[137,137,138] and the cotton rat (*Sigmodon*

hispidus)[129,139,140] have been used extensively to model RSV disease, ERD, and vaccine immunogenicity and efficacy. The major advantages of using mice over cotton rats include the availability of knock-out strains and the availability of a large array of reagents for immunological assessment.[134] Advantages of the cotton rat include the increased permissiveness and the more faithful replication of ERD-associated lung pathology after immunization with FI-RSV and subsequent infection.[141] These two animal models are therefore, highly suitable for use in preclinical evaluation of RSV vaccines such as, for instance, an adjuvanted virosomal RSV vaccine.

Aim and outline of the study described in this thesis

This thesis describes the development and preclinical evaluation of a novel virosomal RSV vaccine supplemented with specific TLR-ligands. In **Chapter 2** the feasibility of the production of reconstituted RSV membranes using DCPC is investigated. Also, the incorporation of the TLR2 ligand Pam3CSK4 in virosomal membranes is studied and the immunogenicity, protective efficacy and safety of the virosomal formulation is evaluated in mice and in cotton rats. To investigate the potency of a more widely used TLR4 ligand with potentially stronger Th1-skewing properties, virosomes with incorporated MPLA are tested in **Chapter 3.** TLR-signaling properties of the virosomes are evaluated *in vitro* together with their ability to induce a protective and safe Th1-skewed anti-viral immune response *in vivo* in mice. **Chapter 4** describes the immunogenicity, protective capacity and safety of RSV-MPLA virosomes in cotton rats. Here emphasis lies on analysis of local cytokine production in RSV-infected lungs of vaccine-primed animals and analysis of histopathological events in the lung following infection of cotton rats. In **Chapter 5,** the capacity of the RSV-MPLA virosomal vaccine to induce protective immune responses are investigated in an approach using mucosal immunization, i.e. intranasal immunization, of mice and cotton rats. **Chapter 6** describes the capacity of RSV virosomes, with or without MPLA adjuvant, to induce protective immunity in an aged immune system. Here, aged cotton rats are used as an animal model. Additionally, the capacity of the virosomal RSV vaccine to boost pre-existing immunity in the aged cotton rats is explored. Finally, the results are summarized and discussed in **Chapter 7.** This chapter also presents a future perspective on the use of an adjuvant supplemented virosomal RSV vaccine.

Chapter 2

Lipopeptide-adjuvanted respiratory syncytial virus virosomes: A safe and immunogenic non-replicating vaccine formulation

Toon Stegmann[1,2], Tobias Kamphuis[1], Tjarko Meijerhof[1], Ellen Goud[2], Aalzen de Haan[1], Jan Wilschut[1]

1) Department of Medical Microbiology, Molecular Virology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
2) Mymetics BV Leiden, The Netherlands

Vaccine, 2010 Aug 2;28(34): 5543-50

Abstract

Respiratory syncytial virus (RSV) causes severe respiratory disease in children and the elderly. There is no registered RSV vaccine. Early experimental non-replicating vaccines have been found to exacerbate RSV symptoms upon infection causing enhanced respiratory disease.

Here we show that immunization of mice with reconstituted virosomes produced from RSV envelopes and containing the lipopeptide adjuvant (Pam3CSK4), induces high-titer virus-neutralizing antibodies, and the secretion of IFN-γ through both MHC-I and MHC-II presentation of antigen, with a balanced Th1/Th2 profile. Immunization with RSV-Pam3CSK4 virosomes provides sterilizing immunity to virus challenge in mice and cotton rats, while not producing symptoms of enhanced disease. Therefore, these virosomes represent a promising candidate inactivated RSV vaccine formulation.

Introduction

According to the WHO, respiratory syncytial virus (RSV) causes 64 million infections annually, with an estimated mortality of 160,000,[142] contributing as much as influenza to death from respiratory disease in the elderly,[143] and leading to the hospitalization of 18,000-75,000 children in the USA alone. However, in contrast to influenza, there is no registered vaccine for RSV. Both replicating and non-replicating vaccine formulations are currently being developed. So far, attempts to produce vaccines that are based on replicating attenuated or recombinant virus have resulted in overattenuation or in vaccines that are not sufficiently tolerated. Development of non-replicating vaccines has been complicated by the disastrous outcome of a clinical trial with a formaldehyde-inactivated, alum-adjuvanted virus vaccine (FI-RSV) in the 1960's. Natural RSV infection in children vaccinated with FI-RSV, that were seronegative prior to vaccination, resulted in enhanced disease symptoms, and two of the children died.[68] Although sera from the vaccinees contained antibodies to the viral F and G membrane proteins, these antibodies did not neutralize the virus. In mice vaccinated with FI-RSV, it has further been demonstrated that enhanced disease was characterized by a response skewed towards Th2 cells,[77] associated with lung eosinophilia and alveolitis. Recent findings have identified crucial factors in the induction of an effective immune response against RSV without priming for enhanced disease. As had long been thought, formaldehyde modification of epitopes in the virus contributes to the production of non-neutralizing antibodies[76] and formaldehyde treatment of vaccines should therefore be avoided. However, some non-formaldehyde-treated non-replicating vaccines are also able to elicit symptoms of enhanced disease.[76,144] More importantly, it was demonstrated that a lack of antibody affinity maturation, caused by poor Toll-like receptor (TLR) stimulation by FI-RSV, or other types of non-replicating vaccines, results in the production of low-avidity antibodies and primes for enhanced disease.[76] The addition of a mixture of TLR-adjuvants to non-formaldehyde-treated non-replicating vaccines induced virus-neutralizing antibodies and prevented enhanced disease.[76] These findings are in line with observations suggesting that addition of monophosphoryl lipid A, a ligand for TLR-4, to FI-RSV attenuates the symptoms of enhanced disease.[78] Priming of mice with live virus prior to FI-RSV vaccination, resulting in overt TLR stimulation, led to a Th1-skewed immune response and prevented the development of enhanced disease.[77] These new insights suggest that a non-replicating RSV vaccine that does not prime for enhanced disease can be made provided it is properly adjuvanted.

Physical association between adjuvant and antigen may ensure activation of antigen-presenting cells (APCs) with TLR activation of that same cell. Virosomes are non-replicating vaccines composed of the reconstituted membrane of enveloped viruses.[106] Virosome vaccines currently in use, or being developed as vaccines are mostly based on influenza virus[106] and are used as a vaccine for influenza virus. They may additionally carry antigens from other pathogens.[108] When properly reconstituted, influenza-based virosome vaccines retain the receptor binding and membrane fusion activities of influenza virus enabling the virosomes to enter APCs by

receptor-mediated endocytosis. Virosome-endosome fusion then follows and the antigen is delivered to the cytoplasm of the cell.[145] The antigen delivered by virosomes is then degraded by cytosolic proteasomes and the products are transported, through TAP, to the ER where they form complexes with MHC-I molecules. This leads to class I MHC-restricted Ag presentation and a cellular immune response, which includes cytotoxic T cell responses. Since APCs take up particles more efficiently than non-particulate antigen, the virosomes augment the immune response to the antigen. To further enhance the adjuvant effect of virosomes, TLR-agonists can be incorporated in their membrane activating the immune response by cells that take up the virosomes.[146,147] Influenza virosomes containing the F protein of RSV, and adjuvanted with the *E. coli* heat-labile toxin (HLT), have previously been tested as an experimental RSV vaccine formulation. However, the immune response to these virosomes was characterized by > 20:1 ratio of IgG1 to IgG2a, representing Th2- and Th1-signature isotype antibodies respectively, indicating that the immune response was biased toward a Th2-type response and afforded the vaccinated animals only a one log protection against a virus challenge.[148]

Here we have produced virosomes directly from the RSV envelope using a reconstitution method that does not involve detergent but, instead, the short-chain phospholipid 1,2 dihexanoyl-sn-glycero-3-phosphocholine (DCPC).[111] The virosomes were adjuvanted with the synthetic lipopeptide adjuvant Pam3CSK4 that is recognized by TLR-2.[149] It is demonstrated that these virosomes induce a protective immune response to the virus with a balanced Th1/Th2 profile, without the immunological correlates of enhanced disease.

Results

Reconstitution of RSV envelopes and incorporation of lipopeptide

For the preparation of RSV-derived virosomes, we solubilized RSV with the short-chain phospholipid DCPC and subsequently reconstituted viral envelopes, using a method developed for influenza virus.[111] This method, after the solubilization step, involves centrifugation to remove the viral nucleocapsid and dialysis of the supernatant to remove the DCPC, resulting in the formation of virosomes. Solubilization of BPL-inactivated RSV, strain A2, using 100 mM DCPC disrupted the viral membrane. After solubilization and ultracentrifugation the viral membrane glycoproteins, F and G, were found in the supernatant and not in the pellet (periodic acid-Schiff stained SDS-PAGE gels, not shown). The supernatant contained about 30% of the total viral protein. The viral supernatant was mixed with additional dissolved lipids in DCPC. Subsequent dialysis of the DCPC from the lipid/supernatant mixture resulted in particles migrating as a single peak of protein and lipid at a density of about 1.12 g/ml, indicating that virosomes were formed (Figure 2.1A). Adding the lipopeptide Pam3CSK4 to the supernatant along with the lipids also resulted in a similar peak of virosomes (Figure 2.1B). Two different lipid mixtures were found to support particle formation. First a mixture of phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), sphingomyelin (SM) and cholesterol at a molar ratio of 2:1:3.5:3.5 (Figure 2.1A), which had previously been found crucial for the proper reconstitution of another paramyxovirus, Sendai virus.[150] Second a mixture of PC and PE, at a molar ratio of 2:1 (not shown). The ratio of lipid to protein in the supernatant/lipid mixture could be varied, yielding virosomes with different protein/lipid ratios.

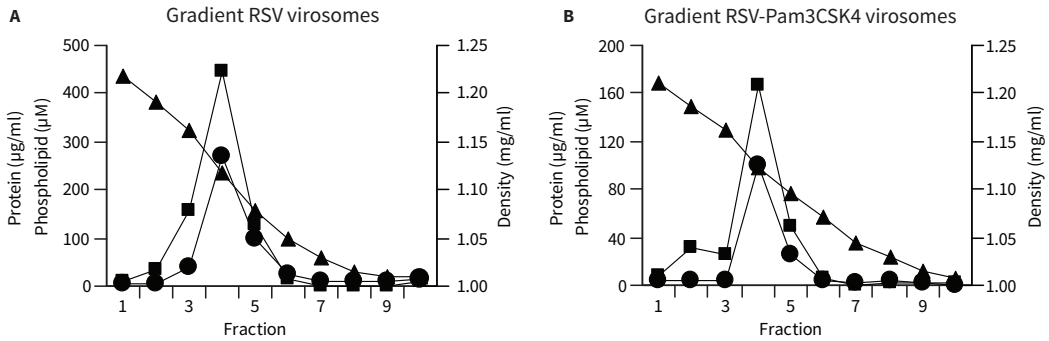


Figure 2.1. Equilibrium density sucrose gradient analyses of dialyzed viral supernatant samples. A) With added PC/PE/SM/Chol, supernatant protein/lipid ratio 1 mg/0.3 µmol. B) With added PC/PE/SM/Chol and Pam3CSK4 protein/ lipopeptide/ lipid ratio 1 mg/ 1mg/ 0.8 µmol

Negative stain electron microscopy of the virosomes showed vesicles of 70-130 nm in diameter, with the viral glycoproteins forming a fuzzy white ring of spikes around the vesicles (Figure 2.2A). The virosomes were shown to contain the viral membrane proteins F and G and to some extent protein M. The viral proteins N and P were not present, indicating the effective removal of viral nucleocapsid components (Figure 2.2B). The lipopeptide adjuvant was present within the peak

fraction of the virosomes on the sucrose gradients, and was therefore most likely incorporated in the virosomes (Figure 2.2B).

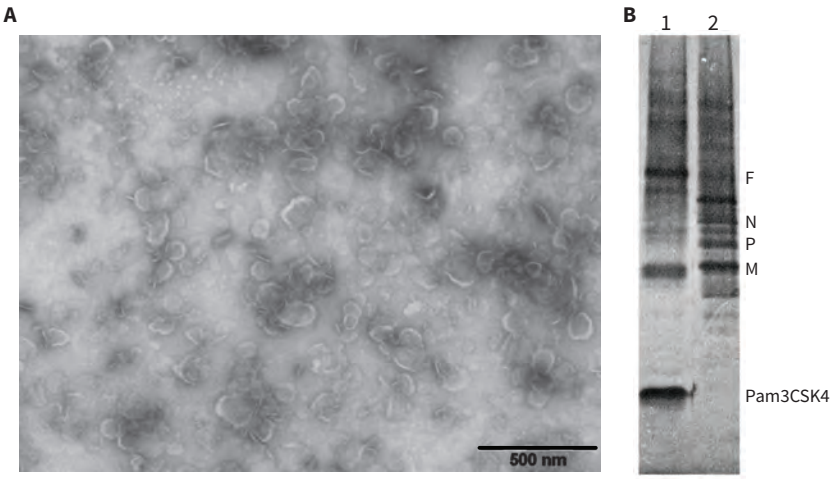


Figure 2.2. Electron microscopy and virosomes proteins. A) Electron micrograph of virosomes. Bar is 200 nm . B) Silver-stained SDS-PAGE gel of virosomes (lane 1) and virus (lane 2) under non-reducing conditions (protein G does not stain well with silver stain). The virosomes are those of the peak fraction, in Figure 1.1B.

Immunization of mice with RSV virosomes

Female BALB/c mice were immunized once or twice, at a 2-week interval, by i.m. injections of viral protein with 5 µg per injection, using virosomes with or without the Pam3CSK4 adjuvant. Blood samples were taken 2 weeks after injection. A single injection with RSV virosomes induced a (10 log) titer of 2.8 ± 0.3, whereas a single injection with virosomes containing the adjuvant resulted in a significantly higher ($p < 0.001$) titer of 3.9 ± 0.1 (Figure 2.3A). A second injection raised these titers to 3.6 ± 0.3 and 4.7 ± 0.3, respectively ($p < 0.001$). On Western blots, sera from immunized mice recognized viral F and G proteins (not shown). To assess the Th1/Th2 skewing of the response, the amounts of IgG1 and IgG2a isotypes were determined, IgG2a being a hallmark Th1 isotype. After two vaccinations the virosomes without adjuvant induced very little IgG2a while vaccination with RSV-Pam3CSK4 virosomes led to a more balanced response (Figure 2.3B). Upon subsequent challenge with live RSV virus this pattern did not change (not shown).

The virus-neutralizing capacity of these antibodies was then tested *ex vivo*. High titers of neutralizing antibodies could be demonstrated, with the adjuvanted virosomes inducing five-fold ($p < 0.01$) higher titers than the virosomes without adjuvant (Figure 2.3C). To test for virus neutralization by the vaccinated animals *in vivo*, mice were vaccinated twice with virosomes with or without Pam3CSK4 as above and challenged with live virus. All vaccinated animals cleared the virus completely after 4 days (Figure 2.3D).

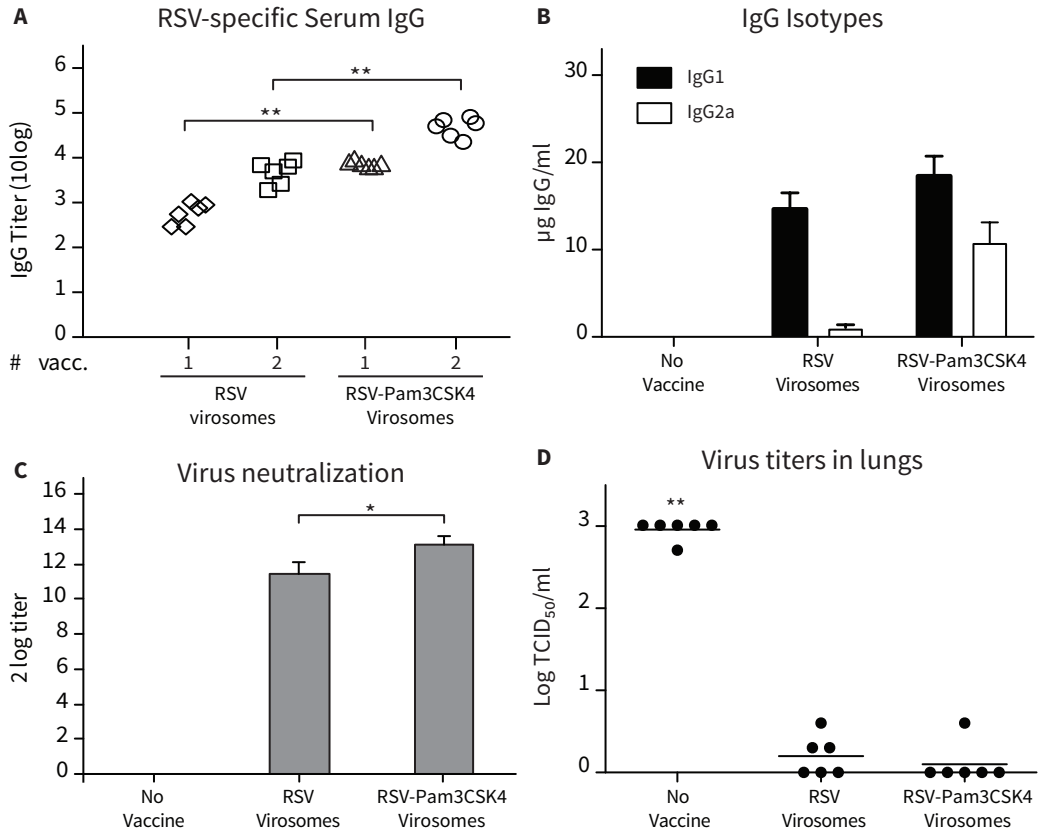


Figure 2.3. Humoral immune response and protection in mice. A) Mice were vaccinated with virosomes with or without Pam3CSK4, either by a single injection with 5 µg of vaccine (lane 1), or two vaccinations at a two week interval (lane 2). B) IgG isotypes in serum were determined for mice which were vaccinated twice at a two week interval, two weeks after the second vaccination by means of ELISA and quantified using a standard curve (bars represent the mean + SEM, ** p<0.001 in Mann-Whitney U test, n=6). C) Serum samples were taken two weeks after the last immunization, Dilutions of the samples were mixed with 100 $TCID_{50}$ of virus, incubated for one hour, after which a TCID50 assay was performed using the mixture. Titers are the reciprocal dilutions giving 50% inhibition of infectivity (bars represent mean + SEM). D) Mice were challenged two weeks after they received their second vaccination with 2.2 x 106 $TCID_{50}$ RSV virus. Four days after challenge, virus titers were determined in 50 µl of minced lung tissue with TCID50. (* p<0.01, ** P<0.001 in Mann-Whitney U test, n=6)

We subsequently analyzed secretion of IFN-γ and IL-5 by spleen cells isolated from these virosome-vaccinated mice challenged with live virus. Spleen cells were stimulated ex vivo with inactivated virus, which results mostly in the presentation of viral antigen via MHC-II molecules and the secretion of IFN-γ, which is characteristic of a Th1 response, while IL-5 secretion is indicative of a Th2 response. In response to the challenge, unvaccinated mice produced some IFN-γ 4 days after challenge and secretion was not significantly enhanced as a result of vaccination with virosomes without Pam3CSK4. However, virosomes with the adjuvant induced 3 times more IFN-γ producing cells ($p < 0.001$; Figure 2.4A). In contrast, the IL-5 secretion that was seen after challenge in unvaccinated mice doubled if the mice had been vaccinated with virosomes, irrespective of whether the virosomes contained adjuvant or not ($p < 0.01$, Figure

2.4B). In conclusion, virosomes with or without adjuvant induce sterilizing immunity in mice but the inclusion of the Pam3CSK4 adjuvant leads to a significant increase in virus-specific, neutralizing antibody titer, significantly promotes the secretion of IFN-γ from RSV-specific T-cells and, most importantly, tends to shift the Th1/Th2 balance towards a Th1 response.

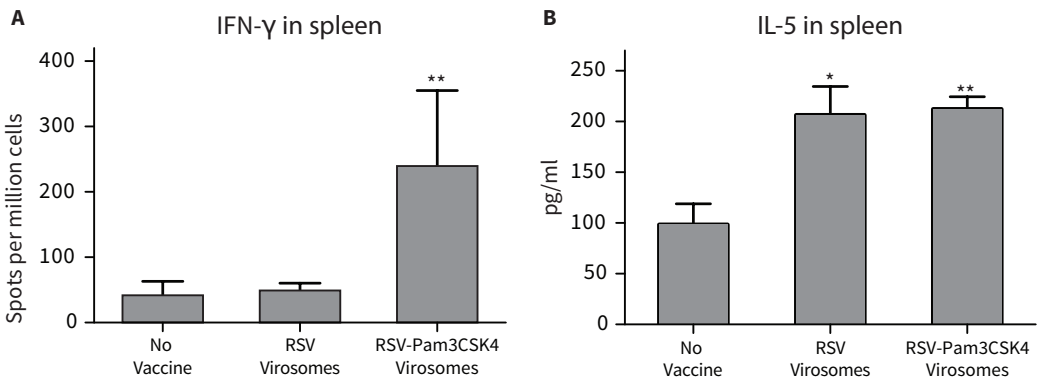


Figure 2.4. Cellular immune response. A) ELISPOT IFN-γ secretion from spleen cells stimulated in vitro with inactivated virus. B) IL-5 secretion (ELISA) from spleen cell cultures stimulated in vitro with inactivated virus (Bars represent mean + SEM, * p<0.01, ** P<0.001 in Mann-Whitney U test, n=6)

Since enhanced disease correlates with an immune response that is skewed towards Th2, our data suggest that the protection against live virus challenge afforded by the adjuvanted virosomes might have occurred without induction of responses associated with enhanced disease. To exanimate this directly the lungs of mice vaccinated with adjuvanted virosomes and subsequently challenged with live virus were examined by histopathological analysis of Giemsa stained lung slices, and compared to those of non-vaccinated mice that had been challenged. None of the mice showed signs of enhanced disease, such as alveolitis, infiltrates rich in eosinophiles, or bronchitis (Figure 2.5).

Immunization of cotton rats

BALB/c mice are relatively sensitive to RSV, inherently biased to a Th2 response, and the role of T-cells in BALB/c pathology induced by RSV has been well characterized.[151] Therefore, the balanced nature of the immune response and the lack of enhanced disease induced by adjuvanted virosomes in mice were encouraging. However, enhanced disease is best studied in cotton rats, since in these animals the lung histopathology can be reproduced that was seen in children after vaccination with the “lot 100” formaldehyde/alum vaccine and subsequent infection with RSV.[140] To produce an FI-RSV vaccine similar to the “lot 100” vaccine, a preparation of RSV A2 virus was inactivated with formaldehyde and precipitated with aluminum hydroxide, according to the protocol for the production of the vaccine as reported by Prince et al.[140] Cotton rats were then vaccinated twice at an interval of 2 weeks with this FI-RSV vaccine preparation or virosomes containing Pam3CSK4. Both vaccine preparations protected the cotton rats against an infectious virus challenge 2 weeks after the last vaccination ($p < 0.001$; Figure 2.6A). This

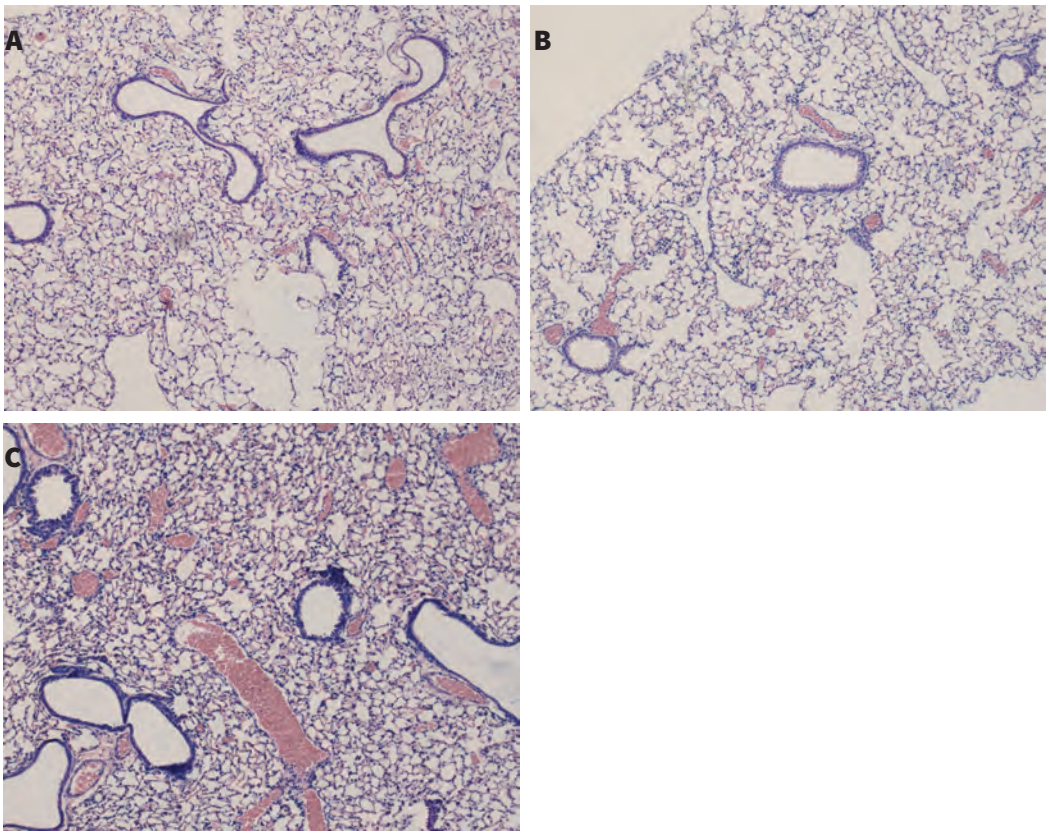


Figure 2.5. Low magnification Giemsa stained micrographs of mouse lungs. A): Mice were vaccinated and challenged as in Figure 2.3, and the lungs were examined 4 days after challenge. (B): Age-matched non-vaccinated, non-challenged mouse.

protective effect of the FI-RSV vaccine in cotton rats is clearly different from the situation in humans, and has been reported before.[152] During the challenge, cotton rats vaccinated with virosomes containing the adjuvant gained more weight than the non-vaccinated group, indicating the challenge had a bigger burden on the non-vaccinated cotton rats ($p < 0.01$; Figure 2.6B). Five days after challenge, the lungs of FI-RSV vaccinated cotton rats showed massive peribronchiolar and perivascular infiltrates containing lymphocytes, eosinophiles and mast cells or macrophages, and clear signs of alveolitis characterized predominantly by intra- and peri-alveolar eosinophiles (Figure 2.7). In contrast, no signs of aveolitis and fewer or no mast cells and macrophages were present in the lungs of unvaccinated and virosome-vaccinated cotton rats after virus challenge. There were limited cell infiltrates containing predominantly lymphocytes. In conclusion, lipopeptide-containing RSV virosomes induce protective immunity in mice and cotton rats, without priming for enhanced disease upon a live virus challenge.

Virosomes containing Pam3CSK4 were also prepared from a B-strain of RSV, and the results of vaccination and the effect of Pam3CSK4 were similar (results not shown).

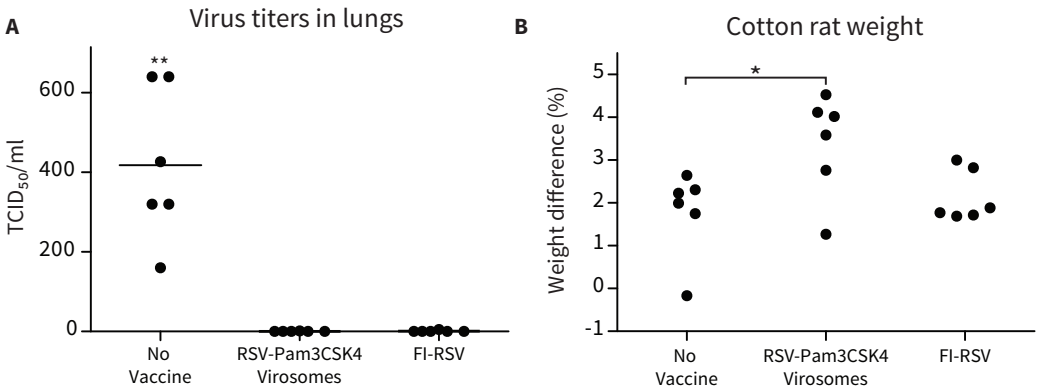


Figure 2.6. Weights and virus titers after vaccination and challenge of cotton rats. Cotton rats were vaccinated twice either with virosomes containing Pam3CSK4 adjuvant, as described in Fig 2.3, or with FI-RSV as described, and challenged with 2.2 x 106 TCID50 infectious virus intranasally, three weeks after the last vaccination. A) the cotton rats were weighed before and after the challenge. The relative increase in weight was plotted for individual mice. B) Four days after the challenge, lungs were removed and $TCID_{50}$ virus titers determined in 50 µl samples of minced lung tissue supernatant. (* $p<0.01$ in Mann-Whitney U test, n=6)

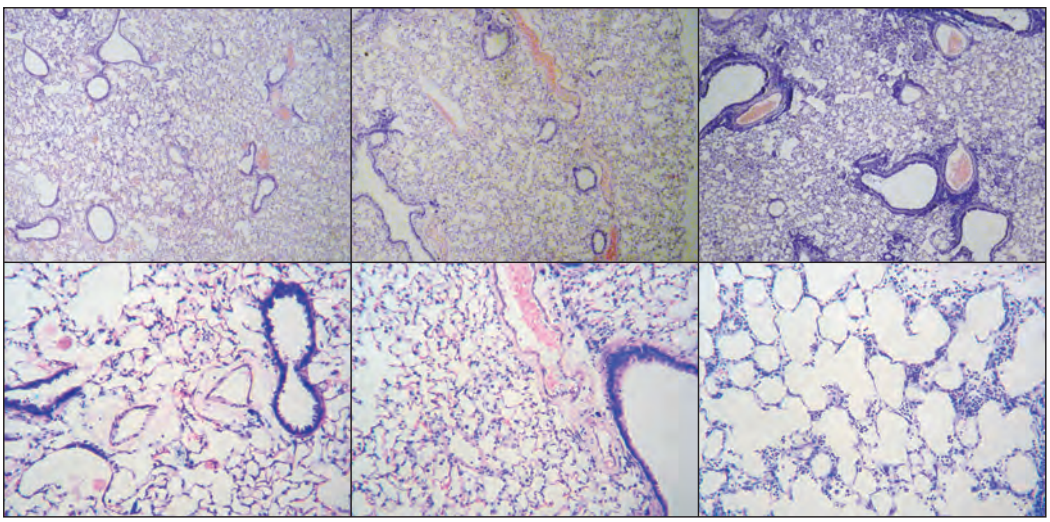


Figure 2.7. Giemsa stained micrographs of cotton rat lungs. Upper row; low magnification, lower row; higher magnification focusing on the alveoli. Cotton rats were vaccinated twice either with virosomes containing Pam3CSK4 adjuvant, as described in Fig 2.3, and challenged with 2.2 x 106 TCID50 infectious virus intranasally, three weeks after the last vaccination. Notice the peribronchiolar en perivascular infiltrates in the FI-RSV vaccinated animals, the absence of these infiltrates in the other animals, and the clear alveolitis in the FI-RSV vaccinated animals.

Discussion

Until recently, it would appear that immunization with RSV proteins or peptides inevitably primes for enhanced disease upon live virus challenge, whereas infection with RSV, either natural or attenuated, or by recombinant viruses expressing RSV proteins, does not lead to enhanced disease upon viral (re)infection. Various hypotheses have been advanced to explain the phenomenon. Most recently, it was demonstrated that a lack of antibody affinity maturation, caused by poor Toll-like receptor (TLR) stimulation by FI-RSV, resulted in the production of low-avidity antibodies to protective epitopes, resulting in a lack of protection.[76] This is in line with previous observations showing that the symptoms of enhanced disease caused by FI-RSV could be attenuated by co-injecting a TLR-4 adjuvant with the vaccine.[78] These data suggest that adjuvanted inactivated vaccines might avoid priming for enhanced disease, provided the immune system is stimulated appropriately.

Here, a vaccine was tested that combines two adjuvanting principles; the presence of multiple antigens in one particle, the virosome, consisting of the reconstituted envelope of RSV and in addition, the presence within its membrane, of Pam3CSK4, an adjuvant recognized by TLR-2. Immunization of mice with these virosomes induced high-titer neutralizing antibodies to the virus, the secretion of IFN-**γ** and a balanced Th1/Th2 profile in mice. Immunization with adjuvanted RSV virosomes also provides sterilizing immunity to virus challenge in mice and cotton rats, without producing symptoms of enhanced disease. Therefore, these virosomes represent a promising, inactivated, candidate RSV vaccine.

Previously, virosomes consisting of reconstituted influenza virus envelopes, containing the purified F protein of RSV and reconstituted using the detergent octaethylene-glycol-mono-*N*-dodecylether with *E. coli* HLT added as an adjuvant have been tested as an RSV vaccine. It was found that these virosomes offered little protection against a live virus challenge, and induced an immune response dominated by IgG1, indicative of a Th2 bias.[148] In our present study, virosomes consisting of reconstituted RSV membranes, without adjuvant, induced sterilizing immunity to RSV challenge, suggesting that the presence of the RSV protein G along with F in the virosomal membrane, the reconstitution by means of the short-chain phospholipid DCPC, or the absence of influenza proteins in the virosomes improves the outcome. In this respect, it has been shown that concomitant vaccination of elderly people with a preparation of the RSV F, G and M proteins and trivalent influenza vaccine had no effect on influenza immunity,[153] indicating that, in humans, RSV proteins do not affect responses to influenza proteins. Conversely, it might be deduced that the presence of influenza proteins *per se* does not affect the response to RSV antigens. However, the presence of influenza proteins in the membrane of these hybrid virosomes,[148] could interfere with the oligomerization of RSV F protein which is required for induction of highly neutralizing antibodies,[154] possibly explaining the superior activity of our virosomes lacking influenza proteins. Also the presence of protein G, in addition to F may have played a role despite the fact that protein F contributes more to RSV immunity than protein G.[155]

Finally, the use of a short-chain phospholipid (DCPC) rather than a detergent and the method applied to remove the DCPC from may have contributed to the favorable outcome of our experiments. Reconstitution of membrane proteins with enzymatic activity using short-chain phospholipids, preserved the activity of the proteins better than detergents.[156,157] Furthermore, the currently used method preserves the fusion activity of the influenza hemagglutinin protein after reconstitution very well.[111] This data suggests that our method of reconstitution optimally preserves protein conformation and, consequently, contributes to optimal induction of protective antibodies.

Comparing the activity of the DCPC-reconstituted virosomes, with or without Pam3CSK4 adjuvant, it was found that both types of virosomes induced sterilizing immunity to virus challenge in mice. However, the adjuvanted virosomes induced significantly higher antibody titers, more effective neutralizing antibodies and more IFN-γ production, while not inducing the secretion of more IL-5 from MHC-II presented antigen, suggesting that the adjuvant had a beneficial effect on the Th1/Th2 ratio.

Therefore, the inclusion of the adjuvant in the virosomal membrane merits consideration in future clinical trials. The choice of adjuvants for any human vaccine remains empirical because so far, very few adjuvants have been allowed in vaccines. Aluminum phosphate did not enhance the immune response to a preparation of RSV F, G, and M protein in clinical trials.[158] The lipopeptide Pam3CSK4 is synthetic and has an excellent safety profile in animals. Its inclusion in the virosomal membranes means that minimal concentrations can be used. Other hydrophobic adjuvants, such as monophosphoryl lipid A, a component of other human vaccines, may also be included in the virosomal membrane, and could improve the safety profile and/or immunogenicity of virosomal RSV vaccines even further.

Materials and methods

Virus and virosomes

RSV strains A2 (American Type Culture Collection, ATCC) and strain B1 (National Collection of Pathogenic Viruses, UK) were grown in roller bottles on Hep-2 cells (ATCC), purified by a combination of differential and rate zonal ultracentrifugation on sucrose gradients and stored frozen in 17.4% glycerol, 145 mM NaCl, 2.5 mM HEPES, 0.1 mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.4 (adopted from Gupta *et al*[159]). After rapid thawing, aliquots of the virus were inactivated with 0.025% beta-propiolactone (BPL) (Acros Organics, Amsterdam, The Netherlands), for 16 h at 4°C with gentle shaking, followed by 2 hr of incubation at 37°C to inactivate the BPL. The virus was then pelleted by ultracentrifugation for 20 min at 90000 g, the supernatant removed and the pellets were suspended in sterile buffer containing 5 mM Hepes, 145 mM NaCl, 1 mM EDTA, pH 7.4 (HNE) at 4°C. For reconstitution with DCPC, an equal volume of sterile 200 mM DCPC in HNE was added. The sample was mixed by pipetting and incubated for 45 min at 4°C. To remove the viral nucleocapsids the sample was spun in sterile tabletop ultracentrifuge eppendorf tubes at 90,000 x g for 30 min at 4°C. The supernatant was filtered through a 0.22 µm filter, the volume was measured and the protein concentration was determined by the Bio-Rad Bradford protein assay (Bio-Rad, Veenendaal, The Netherlands). For the addition of lipids, a lipid mix was prepared from stock solutions of egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), with or without brain sphingomyelin (SM) and cholesterol (Chol) (all lipids from Avanti Polar Lipids, Alabaster, AL, USA) in chloroform/methanol 2:1. The molar ratios for PC/PE/SM/Chol and PC/PE mixtures were 2/1/3.5/3.5 and 2/1, respectively. The mixture was evaporated to dryness on the wall of a glass tube and traces of the solvents were removed at a high vacuum. The lipopeptide adjuvant, *N*-palmitoyl-S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-[R]-cysteinyl-[S]-seryl-[S]-$(lysyl)_3$-lysine (Pam3CSK4, EMC Microcollections GmbH, Tübingen, Germany, lyophilized from the HCl solution), was dissolved in 100 mM DCPC in HNE, the pH was adjusted to 7.4 with NaOH, and the solution filtered through an 0.22 µm filter. To prepare virosomes, the viral DCPC supernatant was combined with the thin film of lipids (0.8 µmol per mg protein unless indicated otherwise), and the lipopeptide solution was added in the case of the adjuvanted virosomes (1 mg of lipopeptide per mg of viral protein). The mixture was incubated for 15 min on ice, then filtered through a 0.22 µm filter for sterilization, and DCPC was subsequently removed by dialysis of the mixture against 2 L of HNE buffer pH 7.4 in a sterile slide-a-lyzer (10 kD cut-off; Thermo Scientific, Etten-Leur, The Netherlands) for 48 h. The buffers were changed 4 times. This resulted in the formation of virosomes.

All virosome preparations were found to be sterile after plating on blood/agar plates and incubation for 2 days at 37°C. Virosome formations were analyzed by equilibrium density gradient centrifugation on 10-60% sucrose gradient in HNE buffer, spun for 85 hr in a Beckmann SW55 Ti rotor at 50,000 rpm, and samples from the gradient were analyzed for protein using a Bio-Rad Bradford protein assay (Bio-Rad, Veenendaal, The Netherlands), phospholipid

phosphate as described before[160] and density (by refractometry). Samples were prepared for electron microscopy by application to freshly carbon-coated copper grids, followed by 1% uranyl acetate stain, and air-drying.

FI-RSV was generated as described before.[140] Briefly, African green monkey kidney cells (ATCC) were infected with RSV until CPE was visible in >80% of the cells. The medium was harvested and clarified by low-speed centrifugation (1000 x g, 10 min, 4˚C) and filtered through a 5 µm filter. The filtrate was formalin inactivated for 3 days with stirring and subsequently ultracentrifuged to harvest the inactivated virus. The vaccine was concentrated 25 times and absorbed to 4mg/ml aluminum hydroxide. The compounded material was concentrated 4 times and taken up in medium with 200 units/ml of each neomycin, streptomycin and polymyxin B, and a 1:40,000 final dilution of benzethonium chloride. This yielded a 100 times concentrated vaccine with 16mg/ml alum.

Animals

Animal experiments were evaluated and approved by The Institutional Animal Care and Use Committee of the University of Groningen according to the guidelines provided by Dutch Animal Protection Act. Female specified pathogen-free mice (BALB/c OlaHds, 8-12-week-old) were supplied by Harlan, The Netherlands and kept in individually ventilated cages. Female cotton rats (COT/NCrL, 8-12-week-old) were supplied by Charles River, Germany, and housed conventionally. Intramuscular vaccinations were given on days 0 and 14, under isoflurane anesthesia. In challenge experiments, mice and cotton rats were challenged 2 or 3 weeks after their second vaccination, respectively. For the virus challenge, virus stocks as described above were dialyzed against HNE for 2 h to remove glycerol, animals were anesthetized with 3-4.5% isoflurane/O_2/N_2O, and given 5 x 10 µl of virus (2.2 x 10^6 TCID50) in the nose, at 90s intervals. The cotton rats were weighed before and at the end of the challenge to assess disease severity. At euthanasia, lungs were inflated with PBS, one-half was tied off and removed for virus titration, while the other half was fixed overnight in formaldehyde (4% in PBS), keeping lungs inflated under 20-25 cm water column pressure. The material was embedded in paraffin and 1-2 µm thick lung slices were processed for Giemsa staining. Groups of 6 mice and 6 cotton rats were used unless indicated otherwise.

Immunological assays

For ELISA analysis, serum was harvested, deep frozen, and stored in polypropylene tubes at <-10°C. ELISA plates (Greiner Bio-one, 655092) were coated with RSV at 0.5 µg protein/well in coating buffer (0.05 M carbonate-bicarbonate pH 9.6-9.8) overnight at 37°C. Plates were washed 3 times with coating buffer and blocked with a 2.5% solution of Protifar Plus (Nutricia, The Netherlands) in coating buffer for 45 min at 37°C, then washed twice with coating buffer and 3 times with PBS containing 0.05% Tween20 (Merck 8.22184.1000) (PBST). Serial two-fold dilutions of serum samples were applied to the plates and incubated for 90 min. Plates were washed 3

times with PBST and incubated with a 1:5000 dilution of the appropriate antibody (horseradish-peroxidase-coupled goat anti-mouse IgG, IgG1 or IgG2a; Southern Biotech 1030-05, 1070-05, or 1080-05, respectively) for 60 min at 37°C. Subsequently, the plates were washed 3 times with PBST and 3 times with PBS, drained and filled with staining solution, 20 mg o-phenylene-diamine-dihydrochloride (OPD, Kodak, 1078054), in 50 mM phosphate buffer pH 5.6 with 0.02% µl H_2O_2 for 30 min, after which the reaction was stopped by adding 50 µl 2 M H_2SO_4 per well. The absorption of the solution at 492 nm was determined, and the ELISA titer was defined as the reciprocal of the highest dilution that gave an absorption of 0.2 at 495 nm. For quantification of the IgG1 and IgG2a levels a calibration curve was used. Calibration curves were made as follows. ELISA plates were coated with goat anti-mouse-IgG (heavy and light chains, human absorbed, Southern Biotech, 1031-01) 100 ng/well in coating buffer overnight at 37 ˚C. After washing and blocking of ELISA plates as described above, a stock solution of mouse IgG1 isotype control (Southern Biotech, 1070-01) and mouse IgG2a isotype control (Southern Biotech, 0103-01) was prepared, dilutions were applied to the plates and incubated for 90 min at 37°C, after which the plates were stained as described above.

For ELISPOT assays, ELISA plates were coated with rat anti-mouse IFN-γ (Pharmingen 551216), 0.25 µg per well in PBS for 1 h at 37°C, washed three times with PBST, and incubated with blocking buffer (PBS containing 0.4% BSA). To produce single-cell suspensions, mouse spleens were squashed between sterilized microscope slides and filtered through sterile gauze (150 µm diameter). Cells were collected by centrifugation at 800 x g for 5 min and washed with IDMD medium. Subsequently, erythrocytes were lysed by incubation with hypotonic medium (0.83% NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) for 5 min on ice. The cells were washed with IMDM, and brought to appropriate concentrations. To stimulate the mouse spleen cells, 100 ng/well of BPL-inactivated RSV was added. Blanc control wells received no inactivated virus. Plates prepared as described above were further filled with different concentrations of spleen cells and incubated overnight at 37 ˚C. The medium was then aspirated and the cells were lysed with water. Next, wells were washed with PBST, and then 0.0625 µg/well of biotin-coupled rat anti-mouse IFN-γ Ab (Pharmingen 554410) was added. Plates were incubated for 1 h at 37°C, washed with PBST and incubated with streptavidin-conjugated alkaline phosphatase (Pharmingen 554065). Wells were overlaid with agarose-containing substrate solution and kept in the refrigerator until spots could be counted. The plates were scored manually and blinded. The blank control values were subtracted from the counts. The values were corrected for background spots by subtracting the values of naïve spleen cells, which received inactivated virus stimulation only.

For IL-5 measurements, spleen cells were treated in the same way as for the BPL-inactivated virus restimulated ELISPOT described above. IL-5 secreted to the medium by the cells was determined using an ELISA kit from R & D Biosystems (kit DY405).

Virus titration and neutralization in vitro

To determine the infectious titer of virus, TCID50 titers were determined. In brief, 15,000 Hep-2 cells/well in DMEM containing 2% FCS were seeded in 96-well plates. The next day, wells were incubated with serial dilutions of the virus-containing samples, in triplicate. Plates were incubated for 6 days at 37^{o}C in a 5% CO_2 atmosphere. After incubation, cells were washed twice with PBS, fixed with 1% glutaraldehyde in PBS, stained with Giemsa, and then TCID50 titers were determined. To measure neutralizing antibody titers, serum samples were decomplemented at 56°C for 30 min, serially diluted, mixed with 100 TCID50 of infectious virus, and incubated with Hep-2 for 6 days cells as described above.

Statistical analysis

All statistical analyses were performed with Graphad Prism 5.00 for Mac OSX, (GraphPad Software, San Diego California USA, www.graphpad.com

Chapter 3

Immunogenicity and protective capacity of a virosomal Respiratory Syncytial Virus vaccine adjuvanted with monophosphoryl lipid A in mice

Tobias Kamphuis[1], Tjarko Meijerhof[1], Toon Stegmann[1,2], Julia Lederhofer[1], Jan Wilschut[1], Aalzen de Haan[1]

1) Department of Medical Microbiology, Molecular Virology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
2) Mymetics BV Leiden, The Netherlands

PLoS One. 2012;7(5):e36812

Abstract

Respiratory Syncytial Virus (RSV) is a major cause of viral brochiolitis in infants and young children and is also a significant problem in elderly and immuno-compromised adults. To date there is no efficacious and safe RSV vaccine, partially because of the outcome of a clinical trial in the 1960s with a formalin-inactivated RSV vaccine (FI-RSV). This vaccine caused enhanced respiratory disease upon exposure to the live virus, leading to increased morbidity and the death of two children. Subsequent analyses of this incident showed that FI-RSV induces a Th2-skewed immune response together with poorly neutralizing antibodies. As a new approach, we used reconstituted RSV viral envelopes, i.e. virosomes, with incorporated monophosphoryl lipid A (MPLA) adjuvant to enhance immunogenicity and to skew the immune response towards a Th1-phenotype.

Incorporation of MPLA stimulated the overall immunogenicity of the virosomes compared to non-adjuvanted virosomes in mice. Intramuscular administration of the vaccine led to the induction of RSV-specific IgG2a levels similar to those induced by inoculation of the animals with live RSV. These antibodies were able to neutralize RSV *in vitro*. Furthermore, MPLA-adjuvanted RSV virosomes induced high amounts of IFN-γ and low amounts of IL-5 in both spleens and lungs of immunized and subsequently challenged animals, compared to levels of these cytokines in animals vaccinated with FI-RSV, indicating a Th1-skewed response. Mice vaccinated with RSV-MPLA virosomes were protected from live RSV challenge, clearing the inoculated virus without showing signs of lung pathology. Taken together, these data demonstrate that RSV-MPLA virosomes represent a safe and efficacious vaccine candidate, which warrants further evaluation.

INTRODUCTION

Respiratory Syncytial Virus (RSV) is a major cause of viral brochiolitis in infants and young children and is also a significant problem in elderly and immuno-compromised adults. According to the WHO, annually 64 million people are infected with RSV, and 160,000 people die from the infection around the world.[142] It is estimated that, each year, RSV leads to 3.4 million hospitalizations of children.[2] By the age of two, nearly all children have been infected with RSV. However, natural infection does not evoke long-lasting immunity, which causes people to undergo multiple RSV infections throughout their lives. In healthy adults, RSV infection will manifest itself like a common cold, which is generally cleared within two weeks. When, at old age, the immune system weakens, RSV infections become more severe, leading to, for example, approximately 10,000 deaths in nursing homes in the US each year.[3,161] Current treatment of RSV infection in high-risk infants consists of prophylactic administration of the monoclonal antibody Palivizumab.[55] However, the high costs of monoclonal antibody therapy and the limited duration of efficacy of this treatment warrant the development of an RSV vaccine.[162,163] In elderly, treatment is mainly supportive and consists of administration of fluids, oxygen and antipyretics.[164] Aerosolized Ribavirin is registered for use in some infant groups however, no significant effect has been reported in the elderly.[164]

Even though the need for an RSV vaccine has been recognized for over 60 years, there is currently no licensed RSV vaccine available. This is, in part, due to the disastrous outcome of a clinical trial in the 1960s, which evaluated a formalin-inactivated, alum-adjuvanted, RSV (FI-RSV) vaccine candidate.[67-70] In this trial, children who received the vaccine developed RSV-specific antibodies, but these proved to be poorly virus-neutralizing.[72,73] Instead of preventing infection, vaccination resulted in enhanced respiratory disease (ERD) upon infection with the live virus, leading to a 16-fold increase in hospitalization and even to the death of two children in the vaccinated group.[165] After this trial, many studies have been performed to elucidate the mechanisms causing ERD upon vaccination with FI-RSV and subsequent exposure to live virus. Studies in mice, for example, showed that a Th2-like immune response accompanied by influx of eosinophils into the lungs plays a major role in ERD.[166] Another study in mice has shown that, although FI-RSV does elicit RSV-specific antibodies, these have a limited affinity for neutralizing epitopes on the RSV fusion protein due to lack of affinity maturation.[76] Therefore, a future RSV vaccine should induce a Th1-skewed response together with high levels of strongly neutralizing antibodies.

A promising approach towards the development of vaccines that both skew the immune response to a Th1-type reaction and induce high-affinity antibodies is to include Toll-Like Receptor (TLR) ligands in the vaccine.[167] TLRs recognize Pathogen-Associated Molecular Patterns (PAMPs) from bacteria and viruses and subsequently signal through adaptor molecules such as MyD88 and TRIF to induce the production of inflammatory cytokines and type-I interferons.[114] Activation of TLR4, for example, leads to production of high amounts of IL12 and IFNα resulting in a Th1-

skewed immune response.[40] Importantly, a recent study showed that a UV-inactivated RSV virus, which by itself induces poorly neutralizing antibodies, will give rise to high-affinity and strongly neutralizing antibodies when supplemented with TLR ligands.[76] Using a similar approach, we recently showed that the incorporation of the TLR2 ligand P3CSK4 in an experimental virosomal RSV vaccine promotes the capacity of the vaccine to induce Th1-type cellular responses together with the induction of protective antibodies in mice and cotton rats.[168] Thus, the combination of an RSV vaccine, such as RSV virosomes, with a TLR ligand improves both the immunogenicity and the safety of the vaccine.

Another promising TLR ligand candidate to be used as an adjuvant in an RSV vaccine is the TLR4 ligand monophosphoryl lipid A (MPLA).[169] MPLA is a detoxified derivative of bacterial lipopolysaccharide (LPS).[170] Like LPS, MPLA also signals through TLR4. However, where TLR4 activation by LPS induces signaling through adaptor molecule MyD88, activation by MPLA leads to TRIF-mediated signaling, resulting in enhanced type I IFN production and reduced production of inflammatory cytokines compared to MyD88-mediated signaling.[76,171,172] MPLA stimulates the production of IFNγ by antigen-specific CD4+ T-cells indicating a Th1-skewed response.[127,169] While the TLR2 ligand Pam3CSK4, which we used in our previous study,[168] has been associated with a balanced Th1/Th2-type immune response, MPLA is thus known to induce a significantly Th1-skewed immune response.[173] Furthermore, an MPLA derivative with similar immune-potentiating properties as native MPLA has been evaluated in extensive clinical trials and has shown good efficacy combined with an acceptable safety profile for use in humans when co-administered with a variety of antigens.[128] For these reasons, MPLA is the only TLR ligand which is currently being used as an adjuvant in a number of licensed vaccines.[115,174] Importantly, the addition of MPLA to FI-RSV suppressed the expression of RSV ERD associated cytokines in the lungs of cotton rats.[78] Furthermore, it has been shown that addition of MPLA to FI-RSV promotes the immunogenicity of the vaccine and ameliorates lung pathology after challenge.[129] Thus, the favorable Th1-inducing properties of MPLA, compared to Pam3CSK4, combined with the available data on the inhibitory effects of this TLR ligand on the development of RSV ERD and its acceptable safety profile in humans, led us to explore MPLA as a possible adjuvant in our RSV virosomal vaccine.

We exploited the lipophilic properties of MPLA to incorporate it in the virosomal membrane during the reconstitution process. These virosomes were analyzed for their immunostimulating properties and immunogenicity both *in vitro* and *in vivo* and for their capacity to induce protection against infection with live RSV. Our data show that incorporation of MPLA in RSV virosomes increases their immunostimulatory capacity *in vitro,* as evidenced by increased human TLR4-mediated NF-κB activation and upregulation of costimulatory molecules in mouse dendritic cells. *In vivo*, incorporation of MPLA in RSV virosomes stimulated RSV-specific IgG antibody levels, with increased IgG2a antibody production and increased levels of virus neutralizing antibodies compared to non-adjuvanted RSV virosomes. Also, RSV-MPLA virosomes

primed for Th1-type responses as evidenced by high IFN-γ levels and low IL-5 levels, not only in *ex vivo* cultures of splenocytes from immunized mice stimulated with RSV antigen, but also in the lungs of immunized mice upon challenge with live RSV. Finally, mice vaccinated with RSV-MPLA virosomes were protected from challenge with live RSV without symptoms of ERD, as demonstrated by the absence of lung pathology and a lack of eosinophil infiltration into the lungs.

RESULTS

Characterization of RSV-MPLA virosomes

The formation of virosomes was analyzed by equilibrium density-gradient centrifugation. Protein and phosphate were found to co-migrate for RSV virosome preparations with and without MPLA, indicating successful reconstitution of the viral envelopes (Figure 3.1A, 3.1B). For RSV-MPLA virosomes, the apparent absence of phosphate outside the virosome peak indicated that MPLA was primarily associated with the virosomal membranes.

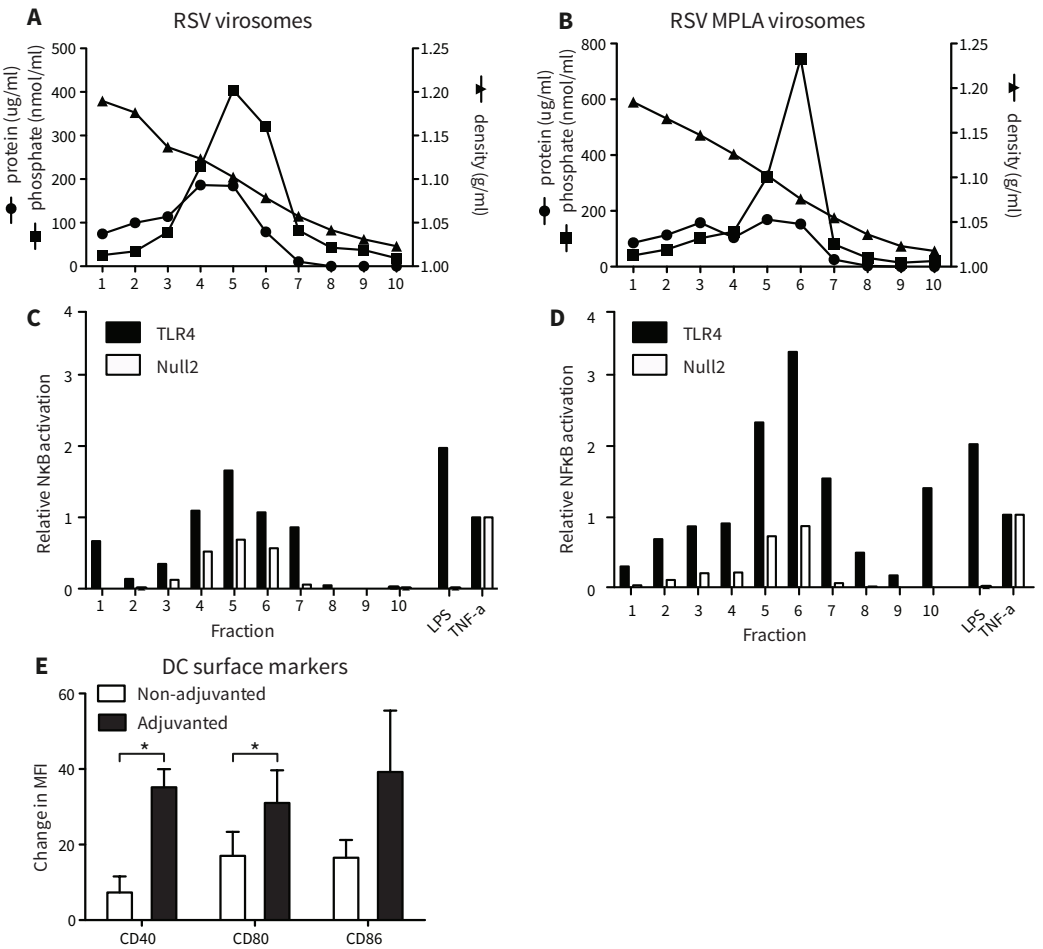


Figure 3.1. In vitro analysis of RSV and RSV-MPLA virosomes. A,B) RSV virosomes and RSV-MPLA virosomes were spun on an equilibrium density sucrose gradient. Subsequently, density, protein concentration, and phosphate concentrations of each fraction was determined. C,D) Fractions from A and B were analyzed for their TLR4-signaling ability using Hek-Blue TLR4 cells. To assess non-TLR specific activation of cells, control cells (Null2 cells) were incubated with the same virosome fractions. As a control for activation both Hek blue TLR4 and Hek blue null2 cells were stimulated with 100 ng/ml TNF-α. Bars represent TLR activation relative to that of the TNF- α control. E) Upregulation of DCs costimulatory molecules CD40, CD86, CD80. Unfractionated virosome preparations were used to stimulate ex vivo cultured mouse DCs overnight. Cells were stained for expression of costimulatory molecules using specific monoclonal antibodies and analyzed by FACS. Bars represent the percentage of positive cells.

In vitro analysis of RSV-MPLA virosomes

To assess the immune-potentiating capacity of the RSV-MPLA virosomes, fractions from the sucrose gradient were tested for their TLR4-activating activity in HEK-Blue TLR4 cells, after dialysis to remove the sucrose. The fractions containing the non-adjuvanted virosomes induced a TLR4-mediated NF-κB activation which was slightly higher than the activation induced by TNF-α (Figure 3.1C). This activation is probably due to TLR signaling of the RSV F protein.[42] Incorporation of MPLA into the virosomes strongly stimulated TLR4 signaling by the virosomes. The fraction at the top of the gradient also induced activation of TLR4, indicating that not all the added MPLA had been inserted in to the viral envelopes (Figure 3.1D). Since a large proportion of the MPLA was associated with the virosomal fraction, as judged by phosphate analysis and TLR4-activating capacity of the fractions of the gradient, subsequent experiments were performed with non-fractionated virosomes.

Next, virosomes were tested for their capacity to up-regulate costimulatory molecules in mouse DCs. Non-adjuvanted virosomes induced the upregulation of DC maturation markers CD40, CD80 and CD86. Incorporation of MPLA in to these virosomes significantly stimulated the induction of CD40 and CD80 expression compared to the induction by RSV virosomes (Figure 3.1E).

In vivo immunogenicity

To analyze the immunogenicity of the virosomes *in vivo*, BALB/c mice were vaccinated twice with RSV virosomes or RSV-MPLA virosomes at a 2-week interval. For comparison, mice were inoculated with live RSV (to induce a Th1-skewed immune response) or vaccinated twice with FI-RSV (to induce a Th2-skewed immune response). Two weeks after the first and second vaccination, blood was drawn and serum IgG titers were determined. After the priming immunization, RSV virosomes induced a mean IgG titer of 2.5 Log GMT. Incorporation of MPLA in to the virosomes resulted in significantly increased IgG levels after both priming and booster immunizations, not only compared to the levels induced by non-adjuvanted RSV virosomes but also to the levels induced by FI-RSV and live virus (Figure 3.2A).

Next, RSV-specific IgG1 and IgG2a subtype levels were determined. RSV-MPLA virosomes induced significantly higher levels of IgG2a compared to non-adjuvanted virosomes, reaching similar levels of RSV-specific IgG2a as seen after live virus inoculation (Figure 3.2B). In parallel with the increased RSV-specific IgG2a responses, increases in RSV-specific IgG1 levels were also noted. Non-adjuvanted RSV virosomes and FI-RSV mainly induced IgG1, indicative of a Th2-type response. Live virus inoculations induced low levels of IgG1 and similar levels of IgG2a, compared to those induced by RSV-MPLA virosomes (Figure 3.2B).

To further characterize the humoral immune response, we determined IgE levels in sera and IgA levels in BAL of immunized mice. IgE was exclusively induced by immunization with FI-RSV, but not by immunization with virosomes or live virus (Figure 3.2C). IgA in BAL was detectable

in mice immunized with FI-RSV (4.6±0.1 Log2 GMT) and live virus (5.6±0.6 Log2 GMT), but not in mice immunized with virosomes. For assessment of the functional capacity of the antibodies, we performed a microneutralization assay. Non-adjuvanted RSV virosomes induced similar neutralizing antibody titers to FI-RSV. Incorporation of MPLA in to the virosomes significantly increased the neutralizing antibody titers to levels similar to those induced by live virus (Figure 3.2D)

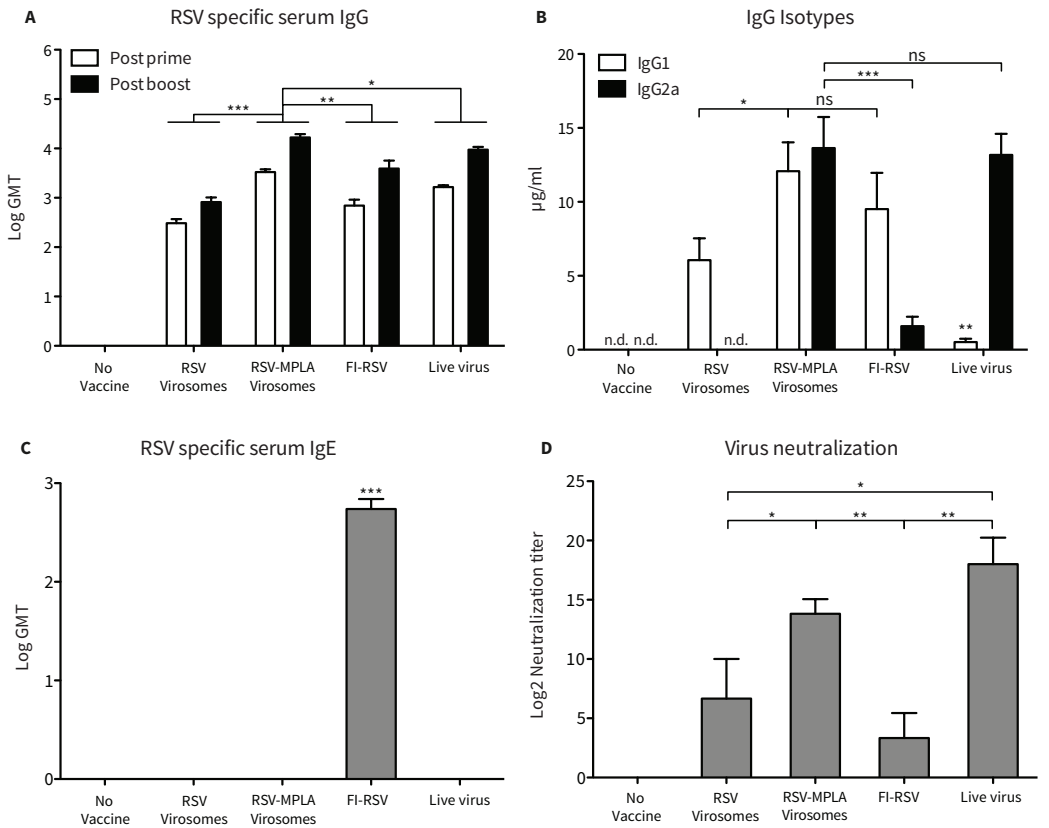


Figure 3.2. RSV specific IgG in mice after vaccination with RSV virosomes and RSV-MPLA virosomes. Mice were vaccinated twice with RSV virosomes, RSV-MPLA virosomes or controls (HNE, live virus and FI-RSV). Each injection contained 5 µg of protein. A) RSV-specific IgG titers in serum 14 days after prime and 14 days after booster vaccination. B) RSV-specific IgG1 and IgG2a subtype levels in serum 14 days after booster vaccination. C) IgE levels were determined at 14 days after booster vaccination. D) RSV neutralizing antibody titers in serum obtained 5 days after challenge. Bars represent the GMT (panels A and C), mean concentration of RSV-specific IgG1/2a (panel B) or mean neutralization titer (panel D) of 6 mice per group. Error bars represent the SEM. Statistical differences were calculated using the Mann-Whitney-U test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Statistical differences in IgE levels were calculated with an ANOVA with Bonferroni correction for multiple testing *** $p<0.001$. The data shown are a representative of two individual experiments

To investigate which concentration of MPLA is needed for optimal adjuvant activity, we added different amounts of MPLA to the viral protein in solution before reconstitution. Apart from the 1:1 protein:MPLA ratio, we also produced virosomes with 1:0.2, 1:0.04 and 1:0.008 protein to MPLA ratios. Using a similar immunization regimen and antigen dose as before, mice were vaccinated,

and RSV-specific serum IgG and subtype responses were determined. The reduction in total RSV-specific serum IgG induced by the vaccine was proportional to the decline in the amount of MPLA in the virosomes (Figure 3.3A). The IgG2a/IgG1 subtype ratio remained similar when the amount of MPLA was reduced from 1 to 0.2 mg/mg protein but decreased when the amount of MPLA was reduced further (Figure 3.3B). This decrease was primarily due to a reduction in RSV-specific IgG2a levels, while the level of RSV-specific IgG1 did not increase significantly with lower amounts of virosome-incorporated MPLA (Figure 3.3C, 3.3D). Because there was no significant difference between the IgG subtypes induced by 1:1 and 1:0.2 protein to MPLA ratio virosomes and there are other benefits to be expected from higher MPLA concentrations (i.e. cellular immune response and reduction in lung pathology) we chose to perform the next experiments with 1:1 protein:MPLA virosomes.

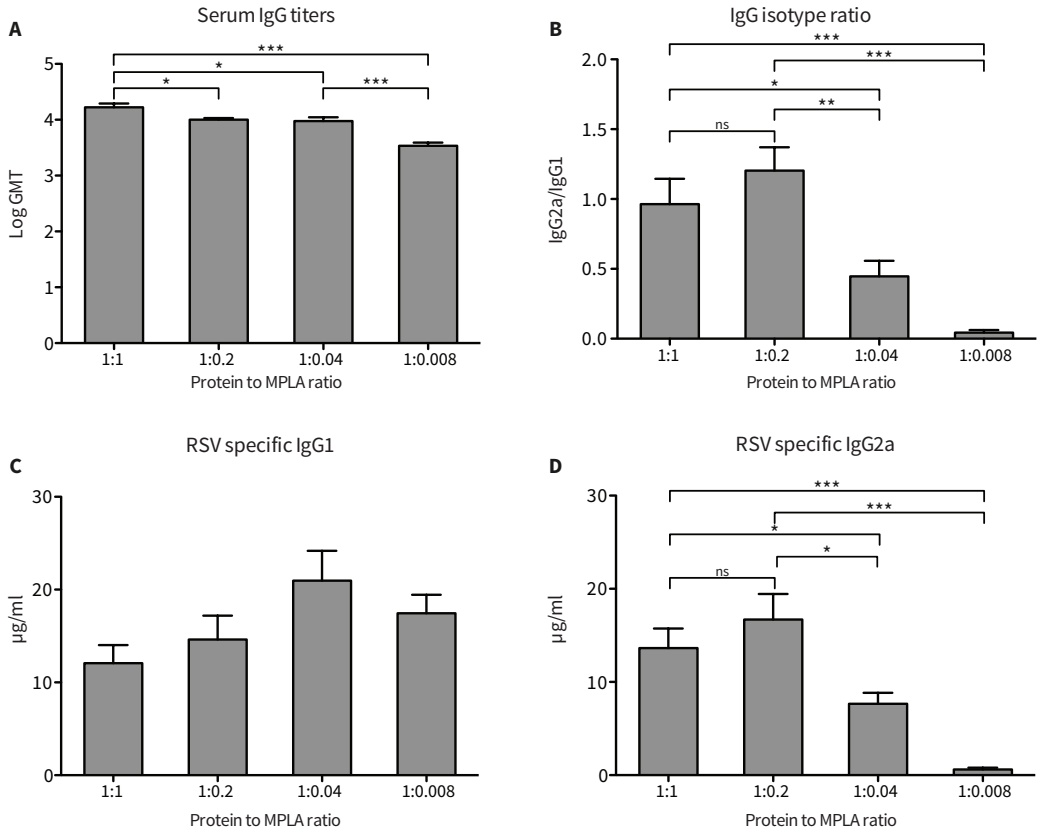


Figure 3.3. Influence of MPLA to virosome protein ratios on RSV specific IgG titers. Mice were vaccinated twice with RSV-MPLA virosomes (5 µg of protein) with different amounts of incorporated MPLA. 14 days after the second vaccination RSV-specific IgG titers in serum were determined. (A) RSV-specific IgG titers. (B) Ratio's of RSV-specific IgG2a/IgG1 concentrations determined 14 days after booster vaccination. (C) RSV-specific IgG1 concentrations. (D) RSV specific IgG2a concentrations. Bars represent the GMT (panel A), mean ratio (panel B) or mean concentration of IgG1/2a of 6 mice per group. Error bars represent the SEM. Statistical differences were calculated using the Mann-Whitney-U test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The data shown are a representative of two individual experiments.

Cellular immunity

To analyze if virosome-incorporated MPLA skews the immune response to a favorable Th1 phenotype, levels of the hallmark Th1 cytokine IFN-γ and Th2 cytokine IL-5 were determined in splenocyte cultures of mice, *ex vivo* stimulated with RSV. Supernatants of splenocytes cultures from mice immunized with RSV-MPLA virosomes or infected with live virus produced significantly increased levels of IFN-γ compared to those from mice immunized with RSV virosomes alone or FI-RSV (Figure 3.4A) Restimulated splenocytes from non-vaccinated mice produced considerable levels of IFN-γ, which may be explained by activation of innate immunity (i.e. NK cell activation) as a result of a high viral load occurring in infected naïve animals. Levels of IL-5 were significantly increased in splenocyte cultures from mice immunized with FI-RSV when compared to those from all other groups (Figure 3.4B).

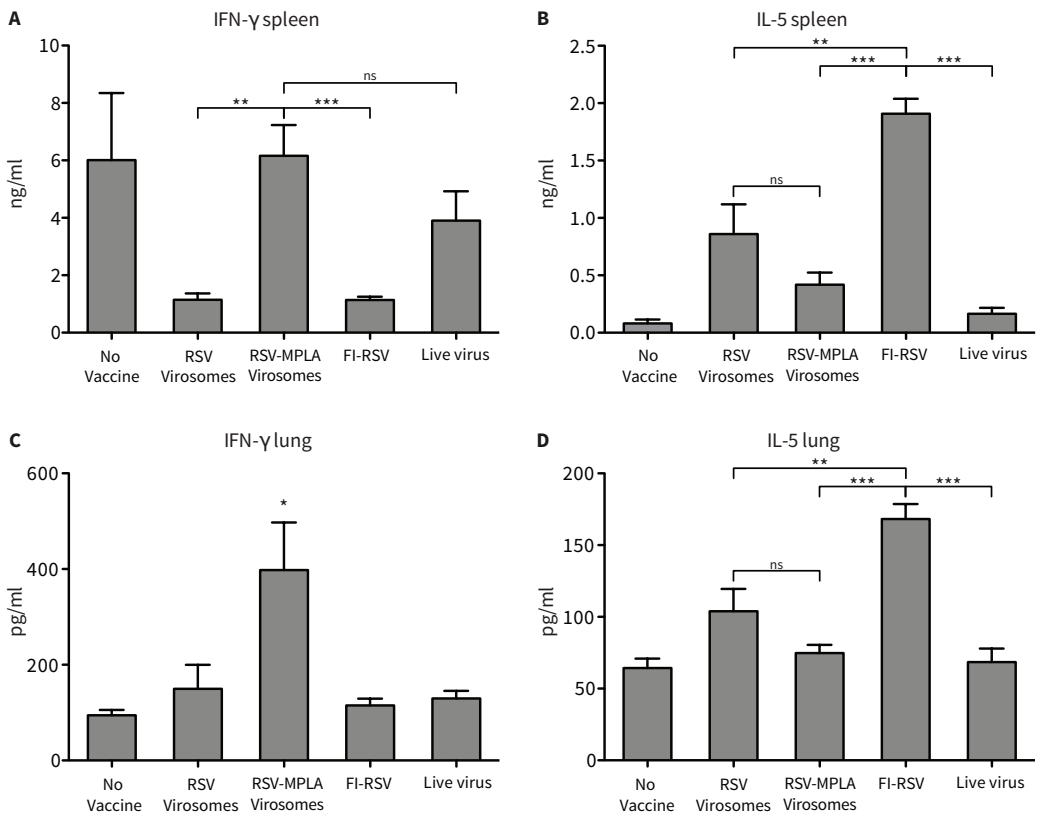


Figure 4. IFN-γ and IL-5 concentrations in RSV-stimulated splenocyte cultures and lung tissue homogenates. Mice were vaccinated twice with RSV virosomes, RSV-MPLA virosomes and control vaccines as in Figure 3.2, and subsequently challenged with live RSV. Four days after challenge, IFN-γ and IL-5 responses were determined. A) IFN-γ concentrations in splenocyte cultures restimulated with BPL-inactivated RSV for three days. B) IL-5 concentrations in splenocyte cultures, restimulated with BPL-inactivated RSV for three days. C) IFN-γ concentrations in homogenated lung tissue, four days after challenge. D) IL-5 concentrations in homogenated lung tissue, four days after challenge. Bars represent the mean cytokine concentration of 6 mice per group and error bars represent the SEM. Statistical differences were calculated using a Mann-Whitney-U test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. The data shown are a representative of two individual experiments.

Next, secretion of these cytokines was measured locally, *i.e.* in lung homogenates, 4 days after viral challenge. In line with the above data, mice immunized with RSV-MPLA virosomes showed significantly increased IFN-γ levels in their lungs upon live virus challenge when compared to levels measured in the lungs of mice immunized with non-adjuvanted virosomes, FI-RSV or live virus immunization (Figure 3.4C). Also, IL-5 levels were significantly increased in the lungs of FI-RSV immunized mice when compared to the levels measured in the lungs of mice immunized with (adjuvanted) RSV virosomes or live virus (Figure 3.4D).

Virus clearance after challenge

To analyze vaccination-induced virus clearance after challenge, mice were immunized twice with HNE buffer, FI-RSV, live virus, RSV virosomes or RSV-MPLA virosomes. Two weeks after the second vaccination mice were challenged with 10^6 $TCID_{50}$ live RSV. Four days later, viral titers were determined in the lungs of the animals. In the HNE vaccinated group, virus was recovered from the lungs of all mice (Figure 3.5). In three out of the six mice immunized with RSV virosomes, virus could not be detected. In the other mice, virus was detected albeit at a significant lower level than in non-immunized mice. In contrast, in all mice immunized with RSV-MPLA virosomes, FI-RSV and live virus, virus could not be detected.

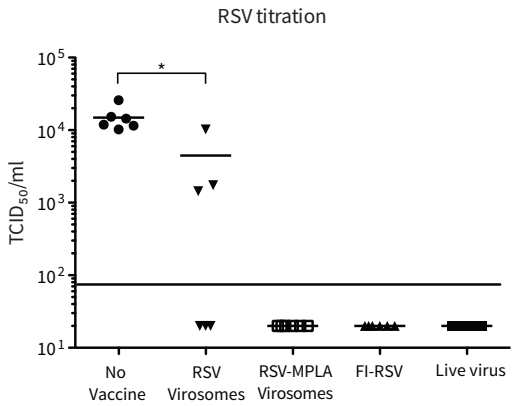


Figure 3.5. Protection against live virus challenge and infiltration of eosinophils. Mice were vaccinated as described in figure 3.2 and challenged with live virus 14 days after the booster vaccination. Four days after challenge, lungs were removed and the viral titer was determined and expressed as TCID50. RSV TCID50 titers from the lungs of challenged animals. Statistical differences were calculated using the Mann-Whitney-U test. * $p<0.05$. The data shown are a representative of two individual experiments

Lung pathology

To further investigate ERD in the immunized mice, we examined lung pathology upon challenge infection (Figure 3.6). Mice immunized with FI-RSV showed signs of alveolitis and infiltrates in both the peribronchial and perivascular areas (Figure 3.6A). The lungs of mice immunized with live virus on the other hand showed no signs of pathology (Figure 3.6B). Mice immunized with RSV virosomes showed no signs of alveolitis but did have perivascular infiltrates (Figure 3.6C) In contrast, the lungs of the mice who received RSV-MPLA virosomes showed no signs of lung pathology (Figure 3.6D) and were very similar to the lungs of mice who received live virus or those of non-immunized mice (Figure 3.6B,E). In addition to this, we assessed the presence of eosinophils in broncho-alveolar lavages (BAL) four days after challenge by May-Grunwald Giemsa

staining of cytospotted cells. No eosinophils were detected in BAL of mice vaccinated with RSV or RSV-MPLA virosomes. On the other hand, in the mice vaccinated with FI-RSV, eosinophils were clearly present (Figure 6F).

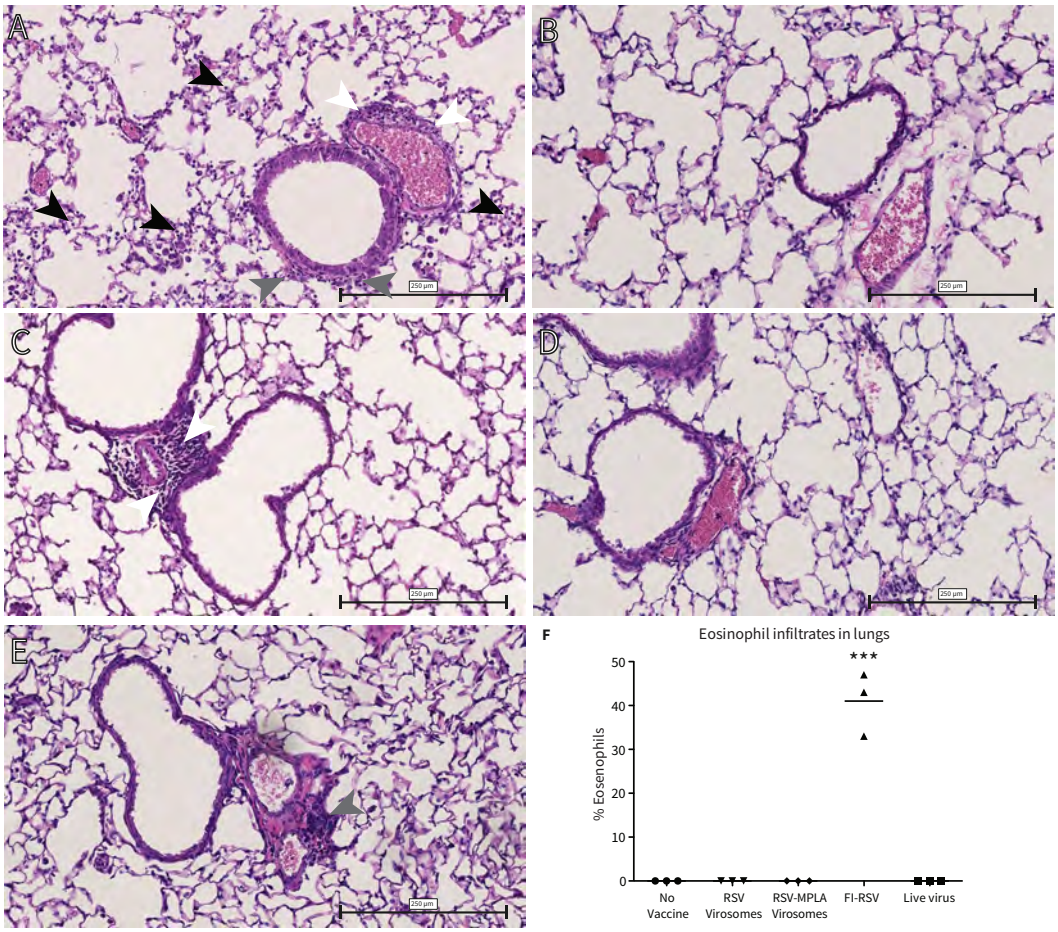


Figure 3.6. Lung pathology in mice after immunization and RSV infection. Mice were immunized and challenged as described in Figure 3.2 and the lungs were harvested, sliced and stained with H&E and assessed for pathology using light microscopy. . Panels represent the lungs of A) FI-RSV, B) live virus, C) RSV virosomes, D) RSV MPLA virosomes E) buffer immunized mice. Black arrows point to alveolar infiltrates, grey arrows to peribronchial infiltrates and white arrows to perivascular infiltrates. F) Eosinophils in BAL expressed as percentage of total BAL cells. Data points represent values from individual mice. Statistical differences were calculated using the ANOVA test with Bonferroni correction for multiple testing. *** $p<0.001$. The data shown are a representative of two individual experiments.

DISCUSSION

Despite the fact that RSV has been recognized as an important vaccine target for more than 60 years, no vaccine is registered for use in humans today. Various vaccine candidates have been evaluated in clinical trials but so far none of them showed the required safety and efficacy profiles. Generally, live attenuated virus vaccines administered intranasally are safe and well tolerated but it is difficult to obtain an optimal balance between immunogenicity and attenuation.[84] Inactivated virus vaccines appear to be hard to advance to the clinic because of the safety concerns related to the outcome of the 1960's FI-RSV trial. Protein subunit vaccines are easy to produce but are generally not very immunogenic and possibly skew towards a Th2 immune response.[84]

In this study, we evaluated the immunogenicity and protective capacity of a virosomal RSV vaccine adjuvanted with MPLA. Incorporation of the TLR4 ligand MPLA into the virosomal membrane resulted in effective human TLR4 stimulation in HEK-Blue cells *in vitro* and activation of mouse DC *ex vivo* as shown by the upregulation of co-stimulatory molecules. Incorporation of MPLA in virosomes resulted in increased RSV-specific serum IgG titers, with production of RSV-specific, Th1-signature, IgG2a-isotype antibodies similar to that induced by live virus inoculation leading to a balanced IgG1/IgG2a profile. These antibodies proved effective in virus neutralization. Furthermore, RSV-MPLA virosomes skewed the cellular responses towards a Th1 profile, as shown by enhanced IFN-γ secretion, not only in *ex vivo* RSV-stimulated splenocytes, but also locally in the lungs of infected mice. Immunization with RSV-MPLA virosomes did not induce any detectable IgE in contrast to immunization with FI-RSV. IgE induction is a hallmark of a Th2-skewed allergy-like response, which is implicated in RSV infections and in FI-RSV induced enhanced disease.[41,175,176] MPLA-adjuvanted virosomes, similar to FI-RSV, provided full protection against live RSV infection, but in contrast to FI-RSV, did not lead to signs of ERD, *i.e.* influx of eosinophils in the lungs or induction of lung pathology. Importantly, previous studies in cotton rats showed that addition of MPLA to FI-RSV reduces the induction of ERD by FI-RSV immunization, illustrated by a reduction in lung pathology, an increase in serum virus neutralization titers and a shift from a Th2 -skewed immune response to a balanced immune response.[78,129] Our observations on the immune response induced by MPLA-adjuvanted RSV virosomes in mice are in line with these data and underline that MPLA-adjuvanted RSV virosomes hold promise as a candidate RSV vaccine. Currently, RSV-MPLA virosomes are being evaluated in cotton rats to optimally assess other ERD parameters, such as alveolitis, in more detail.

Our data show that non-adjuvanted RSV virosomes stimulate human TLR4 in HEK-Blue cells and upregulate co-stimulatory molecules in mouse DC and that incorporated MPLA further enhances these effects. TLR4 activation by RSV virosomes without MPLA is likely to be caused by the RSV F protein. RSV F is a known TLR4 agonist that, for example, induces inflammatory cytokines like IL-6 in DC.[42] Interestingly, despite this capacity to stimulate TLR4, RSV virosomes fail to induce Th1-type responses while MPLA, also a TLR4 agonist, effectively stimulates Th1-type responses.

This could be due to differences in the magnitude of stimulation, which is clearly higher for MPLA (Figure 1), but could also be caused by recruitment of different adaptor molecules downstream of TLR4 activation. As TLR4 uses both MyD88 and TRIF adaptor molecules, it is possible that MPLA competes with RSV F for TLR4 activation. This competition shifts signaling from RSV F-induced, MyD88-dependent, TLR4 signaling to MPLA-induced, TRIF-dependent, TLR4 signaling, leading to a Th1-skewed immune response induced by RSV-MPLA virosomes compared to non-adjuvanted RSV virosomes.

Apart from its influence on T helper cell differentiation, TLR signaling also has a direct effect on IgG isotype switching.[177] Antibody isotype switching is important, because different immunoglobulin subclasses display differences in their ability to mediate effector responses.[178] In mice, the most effective IgG isotype protecting against viral infections is IgG2a.[179] As stated before, MPLA signals through TLR4 to induce type-I IFNs which stimulate IgG2a production predominantly from follicular B cells.[177] Furthermore, MPLA could also directly activate TLR4 on B cells to facilitate isotype switching, a process that is further augmented by IFNα and T-cell help.[180] Previously, we incorporated TLR2 ligand Pam3CSK4 in RSV virosomes. Pam3CSK4 inclusion also skewed towards a Th1 immune response and increased IgG2a levels compared to non-adjuvanted virosomes. Pam3CSK4 adjuvanted RSV virosomes did however, induce slightly higher IgG1 than IgG2a levels. Incorporation of MPLA in the virosomes induces similar IgG1 and IgG2a levels. The relative increase of IgG2a levels compared to Pam3CSK4-RSV virosomes could be due to increased type-I IFN production induced by MPLA. Since incorporation of MPLA in virosomes increases IgG2a levels compared to non-adjuvanted RSV virosomes or FI-RSV, antibodies induced by RSV-MPLA virosomes may well be more effective in protection against viral infection than antibodies induced by the non-adjuvanted RSV virosomes or FI-RSV.

Production of virosomes does not include the application of cross-linking chemicals for inactivation of the virus. This could well be a major advantage of the use of virosomes compared to other approaches using whole inactivated virus. In this respect, it is important to note that one of the reasons why FI-RSV failed to elicit virus-neutralizing antibodies is that important epitopes on the virus are disrupted by formalin.[80] One of the most important RSV epitopes for neutralizing antibodies is a specific conformational epitope making it very susceptible for alteration by chemical treatments, including inactivation with formalin.[181] During virosome production, inactivation occurs through disruption of the membrane by the short-chain phospholipid DCPC followed by removal of the nucleocapsid. This is then followed by reconstitution of RSV F and G protein in the viral membrane with retention of their native conformation. Following this procedure, RSV virosomes lack viral RNA and thus are fully replication-incompetent.[168] Preliminary data indicate that RSV virosomes expose all of the most important known protective on the RSV F protein, as demonstrated by efficient binding of monoclonal antibodies directed to these epitopes (unpublished results).

In conclusion, our data show the feasibility of producing RSV virosomes that have incorporated MPLA. MPLA improves the immunogenicity of RSV virosomes and skews immune response to a protective, balanced Th1/Th2-type response without priming for adverse immune reactions, such as eosinophil influx into the lung after infection with RSV. These data combined with the favorable safety profile of MPLA, and the fact that MPLA is already licensed for use in human vaccines, make the RSV-MPLA virosomal vaccine a suitable candidate for further evaluation in clinical trials.

MATERIALS AND METHODS

Ethical statement

Animal experiments were evaluated and approved by the Committee for Animal Experimentation (DEC) of the University Medical Center Groningen, according to the guidelines provided by the Dutch Animal Protection Act (permit number DEC 5239A). Immunizations and challenges were conducted under isofluorane anesthesia, and every effort was made to minimize suffering.

Virus and cell culture

RSV strain A2 (ATCC VR1540) was kindly donated by Mymetics BV (Leiden, The Netherlands). The virus was grown in roller bottles on HEp-2 cells (ATCC, CL-23, Wesel, Germany) in HEp-2 medium: DMEM (Invitrogen, Breda, The Netherlands) supplemented with Pen/Strep, L-Glutamine, Sodium bicarbonate, HEPES, Sodium Pyruvate, 1X non-essential Amino Acids (all from Invitrogen) and 10% FBS (Lonza-Biowhittaker, Basel, Switzerland) unless stated otherwise. At 80% CPE (5 days post-infection) the medium was cleared by low-speed centrifugation. Aliquots of the supernatant were snap-frozen in liquid nitrogen, as a source of live virus for immunization and challenge. The remainder of the virus was pelleted by ultracentrifugation and subsequently purified on a sucrose gradient. Purified virus was snap-frozen in liquid nitrogen and stored at -80 °C in 20% sucrose in HNE buffer (5 mM Hepes, 145 mM NaCl, 1 mM EDTA, pH 7.4).

Mouse dendritic cells (DCs) were derived from bone-marrow cultures, as described before.[182] Briefly, both tibia and femurs were flushed with Iscove's modified DMEM (IMDM; Invitrogen,) supplemented with 10% FBS, pen/strep, 0.1% β-mercaptoethanol (Invitrogen). Red blood cells were lysed by incubating the cells with ACK buffer (0.83 % NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) for 5 min on ice. The cells were washed with IMDM medium and incubated in IMDM medium supplemented with 200 ng/ml Fms-like tyrosine kinase 3 ligand (Flt3L)(R&D systems, Abingdon, UK). Medium was replaced after 4 days and dendritic cells were harvested 8 days after initiation of the culture.

HEK-Blue TLR4 and HEK-Blue Null2 cells were purchased from Invivogen (Toulouse, France) and maintained according to the manufacturer's protocol.

Vaccine production

RSV virosomes were generated as described previously.[168] Briefly, purified RSV was pelleted by ultracentrifugation and dissolved in 100mM 1,2 dihexanoyl-*sn*-glycero-3-phosphocholine (DCPC) in HNE buffer. The nucleocapsid was removed by ultracentrifugation. Subsequently, a 2:1 molar mixture of egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL, USA) in 2:1 chloroform/methanol at 850 nmol/mg protein was evaporated to a dry film in a glass tube. The supernatant containing the membrane lipids and proteins was added to the lipid mixture. For incorporation of MPLA, monophosphoryl lipid A from *Salmonella minnesota* Re 595 (Invivogen) was first dissolved in 100 mM DCPC in HNE buffer and then added

to the protein/lipid mixture at 1 mg MPLA/ mg virosomal protein. For the MPLA concentration experiment, MPLA was added in lower ratios i.e. 1:0.2, 1:0.04, 1:0.008 (mg virosomal protein to mg MPLA). The mixture was incubated for 15 min at 4°C, filtered through a 0.22µm filter and dialyzed in a sterile Slide-A-lyzer (10 kD cut-off; Thermo Scientific, Geel, Belgium) against 4 x 2 liters of HNE pH 7.4 for 48 hours. After dialysis, virosomes were kept at 4˚C.

FI-RSV vaccine was produced according to the original protocol, which was used for the 1960's FI-RSV preparation as reported in.[183] FI-RSV was diluted in HNE buffer to contain 5 µg of RSV protein in 25 µl of vaccine.

In vitro analyses

The virosomes were analyzed by equilibrium density gradient centrifugation on 10-60% sucrose gradients in HNE. Gradients were spun for 60 hr in an SW 55 Ti rotor at 50000 rpm and samples from the gradient were analyzed for protein, phospholipid phosphate and density (by refractometry). Each fraction was dialyzed against HNE in a Slide-A-Lyzer MINI Dialysis Device (Thermo Scientific, Geel, Belgium) overnight to remove the sucrose which is toxic for HEK-Blue cells at high concentrations. The samples were corrected for increases in volume due to the dialysis and 20µl volumes of the samples were used to stimulate HEK-Blue TLR4 cells (10^5 cells/well) and HEK blue Null2 cells ($5x10^4$ cells per well) overnight at 37˚C in a 96 well plate in triplicate. To quantify alkaline phosphatase production, 20 µl of HEK-Blue cell supernatant was added to 180 µl Quanti-Blue (Invivogen, Toulouse, France) and incubated for 30 minutes at 37˚C. Absorbance was measured at 630 nm and plotted relative to the activation induced by 100 ng/ml of TNFα.

Upregulation of surface markers was assessed after incubating DCs with different virosome preparations. DCs were incubated at 1×10^6 cells/ml at 37 °C in IMDM medium. The incubation was stopped after 24 hr by washing the cells twice in medium. Expression of surface markers was determined by staining with anti-mouse CD80-PE (12-0801-82, eBioscience, Vienna, Austria) anti-mouse CD86- PE (12-0862-82, eBioscience) and anti-mouse CD40-FITC (11-0402-82, eBioscience) using standard staining protocols, followed by flow-cytometric analysis on a FACSCalibur flow cytometer (BD Bio- sciences, Erembodegem, Belgium).

Animal experiments

Female specified-pathogen-free BALB/c OlaHsd mice (6-8 weeks old)(Harlan, Zeist, The Netherlands) were used for all immunization experiments. For immunization and challenge, mice were anesthetized using 3-4.5% isoflurane in O_2. Mice received RSV virosomes, RSV-MPLA virosomes or FI-RSV intramuscularly in 25 µl HNE. Each preparation contained 5 µg of protein. Control mice received 50 µl ($1*10^6$ $TCID_{50}$) of live RSV, intranasally or 25 µl of HNE intramuscularly. Vaccinations were given on day 0 and day 14. On day 28 mice were challenged with 10^6 $TCID_{50}$ (titrated as described below) of live RSV intranasally. On time points of vaccination and challenge,

blood was drawn by retro-orbital puncture. Four days after challenge, mice were sacrificed and blood was drawn by heart puncture. Spleens were harvested for analysis of RSV-specific T cell cytokine responses and lungs for analysis of pathology, determination of lung cytokines and viral titers, respectively.

Virus titration

Virus titers were determined by titration of the tissue-culture infectious dose ($TCID_{50}$). For challenge virus, initial dilutions of 1:5000 were made in HEp-2 medium without FBS. Serial twofold dilutions of these samples were made in 96-well plates in quadruplicate. 20,000 HEp-2 cells were added to the virus dilutions and incubated for 5 days at 37°C in 5% CO_2. The cells were then fixed with 1% paraformaldehyde in PBS for 45 min, blocked with 2% milk powder (Protifar plus, Nutricia, Zoetermeer, The Netherlands) in PBS for 1 hr and stained with 50µl 1:400 FITC-labeled goat anti-RSV antibody (Meridian life science Inc, Saco, ME, USA) at 37°C overnight. The next day, plates were washed with PBS and analyzed under a fluorescence microscope. Wells were considered positive for infection if one or more fluorescent syncytium was present. Titers were calculated using the Reed & Muench method.

To determine virus titers in the lungs of challenged mice, the lungs were removed aseptically after euthanasia of the mice. Lungs were then homogenized in 1 ml of 2% FBS containing HEp-2 medium using an automated Potter homogenizer Polytron-Aggregate® (Thomas Scientific, Swedesboro, NJ, USA). Next, homogenates were centrifuged at 1400 rpm for 10 min at 4°C, and supernatants, diluted to a 1:5 starting dilution, were used to determine viral titers using the $TCID_{50}$ method as described above.

In vitro neutralization assay

Volumes of 100 µl of serum were heat-inactivated for 30 min at 56°C and subsequently diluted with 150 µl serum-free HEp2 medium. Wells of 96-well plates were filled with 50 µl of serum free HEp2 medium. Fifty µl of diluted serum was applied to the first row of wells in quadruplicate and serial two-fold dilutions were made. Subsequently, 70 $TCID_{50}$ of live RSV was added in 50 µl of serum free HEp2 medium and incubated at 37°C for 2 hr. After incubation, 20,000 HEp2 cells were added per well in 100µl of HEp2 medium with 4% FBS. After 5 days of incubation, the cells were washed, fixed and stained as described above for the virus titration. Neutralization titer was calculated with the Reed & Muench method and is indicated as the reciprocal of the dilution that neutralizes infection in 50% of the wells.

Immunological assays

RSV-specific antibody titers were determined as described before.[168] Briefly, 96-well plates were coated with betapropiolactone-inactivated RSV and then blocked with 2.5% milk powder in coating buffer. Plates were then incubated for 90 min with two-fold serial dilutions of serum or broncho-alveolar lavages (BAL; see below), starting at 1:200 for serum or 1:1 for BAL. After

washing, plates were incubated with a 1:5000 dilution of horseradish-peroxidase-coupled goat anti-mouse IgG, IgG1, IgG2a, IgA, or rat anti-mouse IgE (Southern Biotech 1030-05, 1070-05, 1080-05, 1040-05, 1130-05) for 1 hr, washed again and subsequently stained with *o*-Phenylenediamine (OPD; Sigma-Aldrich, St Louis, MO, USA). After 30 min the staining was stopped by addition of 2 M H_2SO_4 and absorption was measured at 492 nm. For levels of total IgG, geometric mean titers (GMT) were determined. For quantification of IgG1 and IgG2a levels, a calibration curve was used. For this, ELISA plates were coated with goat anti-mouse-IgG (heavy and light chain, human absorbed; Southern Biotech, 1031-01) at 100 ng/well in coating buffer overnight at 37°C. After blocking with 2.5% milk powder, known concentrations of a mouse IgG1 isotype control (Southern Biotech, 1070-01) and mouse IgG2a isotype control (Southern Biotech, 0103-01) were prepared, and applied to the plates. After a 90-min incubation at 37°C, plates were washed and stained as described above.

For analysis of levels of IL-5 and IFN-γ in splenocyte cultures and lung homogenates (see below), mouse IFN-γ and mouse IL-5 high sensitivity ELISA kits (eBioscience) were used according to the manufacturer's instruction.

For the analysis of IFN-γ and IL-5 secretion in the RSV-specific recall responses of splenocytes, spleens were removed four days after challenge and transferred to a 15 ml tube containing IMDM/10% FCS. The spleens were passed through a 70-µm cell strainer (BD Biosciences, Heidelberg, Germany) using sterile 3-mL syringe plungers. Erythrocytes were then lysed by incubating with ACK buffer for 5 min on ice. The cells were washed with medium, counted and seeded at 2×10^6 cells/ml and stimulated with BPL-RSV (10 µg/mL) in IMDM/10% FCS in triplicates and incubated at 37°C in a 5% CO_2 atmosphere for 72 hr. Supernatants were harvested and stored at -20°C until further analysis.

For analysis of IL-5 and IFNγ levels in RSV-infected lungs, lungs were removed from challenged mice and homogenized using the method as described for virus titration (see above). IL-5 and IFN-γ levels were then determined in supernatants of centrifuged lung homogenates.

Lung histopathology

The harvested lung lobes were inflated with 4% formalin in PBS and subsequently embedded in paraffin. Four µm slices were then prepared, and stained with standard hematoxylin and eosin. After staining, lung inflammatory parameters (peribronchiolitis, perivaculitis and alveolitis) were assessed by light microscopic analysis of slides.

Broncho-alveolar lavage cytospins

BAL were taken by rinsing the lungs of the mice with 1 ml of PBS supplemented with protease inhibitors using a winged shielded i.v. catheter (1.3x30mm, BD Utah) inserted, through an incision, in the trachea of euthanized mice. Cells in the BAL were pelleted by low-speed

centrifugation and resuspended in 500 µl PBS. In some cases, the remaining BAL supernatants were used for IgA antibody assessment in ELISA. Subsequently, cells were spotted (300 rpm for 5 min) onto glass slides, air dried, and fixed in 80% methanol/20% PBS (V/V) for 10 min at -20°C. After air-drying, slides were stained for 20 min in May-Grunwald-Giemsa stain (Merck, Darmstadt, Germany), diluted 1:1 in Sørensen's phosphate buffer (0.2 M; pH 6.6). Then, slides were rinsed in Sørensen's phosphate buffer, and incubated for 15 min in Giemsa stain (Merck, Darmstadt, Germany) diluted 1:8 in Sørensen's phosphate buffer. After washing with tap water, slides were air-dried and spots were sealed using cover slides and Kaiser's glycerol (Merck, Darmstadt, Germany). The presence of eosinophils in cytospot BAL cells was analyzed by light microscopy.

Statistical analysis

All statistical analyses were performed with Graphad Prism 5.00 for Mac OSX, (GraphPad Software, San Diego California USA, (www.graphpad.com). Statistical significance was assessed using a Mann-Whitney U test. A P value of 0.05 or lower was considered to represent a significant difference.

Chapter 4

A virosomal Respiratory Syncytial Virus vaccine adjuvanted with MPLA provides protection against viral challenge without priming for enhanced disease in cotton rats

Tobias Kamphuis[1], Toon Stegmann[1,2], Tjarko Meijerhof[1], Jan Wilschut[1], Aalzen de Haan[1]

1) Department of Medical Microbiology, Molecular Virology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
2) Mymetics BV Leiden, The Netherlands

Submitted to Influenza and Other Respiratory Viruses

Abstract

Background

Non-replicating Respiratory Syncytial Virus (RSV) vaccine candidates could potentially prime for enhanced respiratory disease (ERD), a Th2-mediated immune pathology, following RSV infection. Vaccines with built-in immune response modifiers, such as Toll-like receptor (TLR) ligands, may avoid such aberrant imprinting of the immune system.

Methods

We developed reconstituted RSV envelopes (virosomes) with incorporated TLR4 ligand, monophosphoryl lipid A (RSV-MPLA virosomes). Immune responses and lung pathology after vaccination and challenge were investigated in ERD- prone cotton rats, and compared to responses induced by live virus and formaldehyde-inactivated vaccine (FI-RSV), a known cause of ERD upon RSV challenge.

Results

Vaccination with RSV-MPLA virosomes induced higher levels of virus-neutralizing antibodies than FI-RSV or live virus infection and provided protection against infection. FI-RSV, but not RSV-MPLA virosomes, primed for increases in expression of Th2 cytokines IL-4, IL-5, IL-13 and Th1 cytokine IL-1b, 6 h to 5 days after infection. By contrast, RSV-MPLA virosomes induced IFN-γ transcripts to similar levels as induced by live virus. Animals vaccinated with FI-RSV, but not RSV-MPLA virosomes showed alveolitis, with prominent neutrophil influx and peribronchiolar and perivascular infiltrates.

Conclusion

These results show that RSV-MPLA virosomes represent a safe and immunogenic vaccine candidate that warrants evaluation in a clinical setting.

Introduction

Respiratory Syncytial Virus (RSV) has been recognized as an important vaccine target for over 60 years; however, there is no vaccine on the market today. A clinical trial conducted in the 1960s evaluated a formalin-inactivated, alum-absorbed RSV preparation (FI-RSV) as a possible vaccine candidate.[70] Infants vaccinated with FI-RSV who subsequently acquired a natural infection, developed enhanced respiratory disease (ERD), especially in the youngest group of vaccinees who were naïve to RSV prior to vaccination, which led to the death of two of these children.[67,68] In those two infants, severe bronchiolitis and alveolitis were observed with an influx of neutrophils and mononuclear cells into the lungs.[68] Although the vaccine significantly increased RSV-specific antibody titers in 95% to 100% of vaccinees, the induced antibodies failed to neutralize the virus in half of the patients.[72,73] Studies in mice demonstrated that impaired Toll-Like Receptor (TLR) signaling by the vaccine results in low antibody affinity maturation and, consequently, in the formation of poorly virus-neutralizing antibodies.[76] Furthermore, it has been shown that FI-RSV induces a predominantly Th2-skewed immune response.[77,166] Therefore, the objective in the development of non-replicating RSV vaccines is to generate a formulation that induces high titers of neutralizing antibodies, skews towards a Th1-type response and does not induce lung pathology upon natural infection.

One way to induce protective immunity without priming for immunopathology involves addition of a Th1-skewing adjuvant to the vaccine. The detoxified lipopolysaccharide (LPS) derivative monophosphoryl lipid A (MPLA) skews the immune response to a Th1-phenotype through activation of TLR4.[169] Additionally, MPLA has an acceptable safety profile when co-administered with vaccine antigens in multiple clinical trials and is the only TLR ligand currently being used as an adjuvant in licensed human vaccines.[115,128,184] Furthermore, it has been shown that addition of MPLA to FI-RSV alleviates the symptoms of RSV enhanced disease.[78]

In a recent study in mice, we showed that reconstituted RSV viral envelopes (virosomes) containing viral membrane proteins and incorporated MPLA but lacking the nucleocapsid, represent a promising vaccine candidate.[185] Immunization with these RSV-MPLA virosomes led to induction of high levels of neutralizing antibodies and a balanced Th1/Th2-phenotype compared to FI-RSV immunization. Mice immunized with RSV-MPLA virosomes cleared the virus after infection and showed no signs of immunopathology.

Even though mice are a valuable model to assess vaccine-induced immune responses, they are not permissive for RSV replication and display a different form of ERD compared to humans. Infiltration of eosinophils is a hallmark of FI-RSV induced ERD in mice while in the 1960s clinical trial, analyses of autopsy samples indicated excessive infiltrating neutrophils and only scarce influx of eosinophils.[77,183] Cotton rats (*Sigmodon Hispidus),* on the other hand, are more permissive

for RSV than mice and also more faithfully replicate FI-RSV-induced immunopathology in humans.[140,183]

The cotton rat was used for preclinical evaluation of the prophylactic antibody Palivizumab and has become the small-animal model of choice for RSV vaccine development.[186] Recently, key cotton rat cytokine genes were sequenced enabling the analysis of Th1/Th2 cytokine profiles using qPCR.[187] It was shown that immunization of cotton rats with FI-RSV not only induces increased Th2 cytokine expression, but also stimulates expression of several Th1-associated cytokines after live virus challenge.[78] The combination of the permissiveness of the cotton rat for RSV, the occurrence of ERD and the new opportunity to profile Th1/Th2 cytokine responses, makes this animal model very suitable to study the safety and efficacy of RSV-MPLA virosomes.

Here we show that RSV-MPLA virosomes induce a superior immune response compared to FI-RSV or non-adjuvanted RSV virosomes, through the induction of increased virus-neutralizing antibody levels, a balanced Th1/Th2 response and the absence of alveolitis or influx of neutrophils in the lungs after challenge. These results, combined with the responses to immunization we observed in mice, show that RSV-MPLA virosomes represent a safe and immunogenic RSV vaccine candidate that warrants further evaluation in a clinical setting.

Results

Immunogenicity of RSV-MPLA virosomes

were produced and analyzed as described before.[168] To determine the immunogenicity of RSV virosomes and RSV-MPLA virosomes, serum samples from immunized cotton rats, collected on day 21 and 49, were analyzed for RSV-specific IgG antibodies (Figure 4.1A). Immunization with RSV virosomes induced a significantly higher RSV-specific IgG titer in serum than immunization with FI-RSV or live virus. The inclusion of MPLA in the RSV virosomes significantly increased the antibody titer to an approximately 2-fold higher level.

To determine the virus-neutralizing capacity of the RSV-specific serum antibodies, a microneutralization test was performed (Figure 4.1B). Although FI-RSV induced similar levels of RSV-specific antibodies compared to immunization with live virus, the antibodies induced by FI-RSV had a significantly lower capacity to neutralize the virus (Figure 4.1B). Sera from rats immunized with RSV virosomes did not show increased capacity to neutralize the virus compared to sera from FI-RSV immunized animals. Sera from rats immunized with RSV-MPLA virosomes demonstrated significantly higher levels of neutralizing antibodies compared to sera from animals immunized with FI-RSV or non-adjuvanted virosomes.

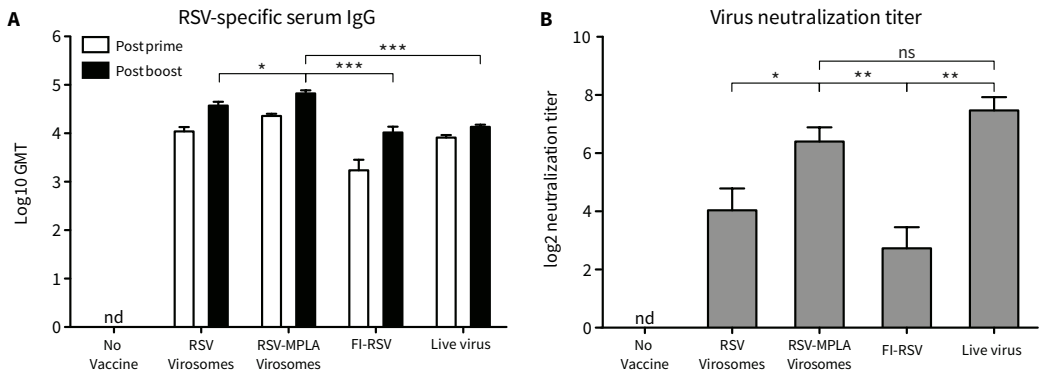


Figure 1. Antibody responses to RSV vaccination. Cotton rats were immunized twice (day 1, prime and 21, boost) with virosomes, FI-RSV or live virus. A) On day 21 and 49, blood was drawn and RSV-specific IgG titers in serum were determined. B) Virus neutralization titer of serum on day 49. (Mann-Whitney U test *p<0.05, **p<0.01, ***p0<0.001)

Protection against RSV infection

To determine whether immunization with MPLA-adjuvanted RSV virosomes could also induce a protective immune response *in vivo*, animals were immunized and challenged with live RSV and lung virus titers were determined (Figure 4.2). At 6 h post-infection, RSV was recovered from 3 out of 5 control animals with titers of approximately 10^2 $TCID_{50}$/gram lung. None of the immunized animals had detectable virus in their lungs at this time point (Figure 4.2A). At 5 days post challenge, increased RSV viral titers were detected in the lungs of all control animals, to above 10^4 $TCID_{50}$/gram lung, indicating virus replication in these animals. In contrast, no RSV was detected in the lungs of the animals immunized with RSV virosomes, RSV-MPLA virosomes, FI-RSV or live virus (Figure 4.2B).

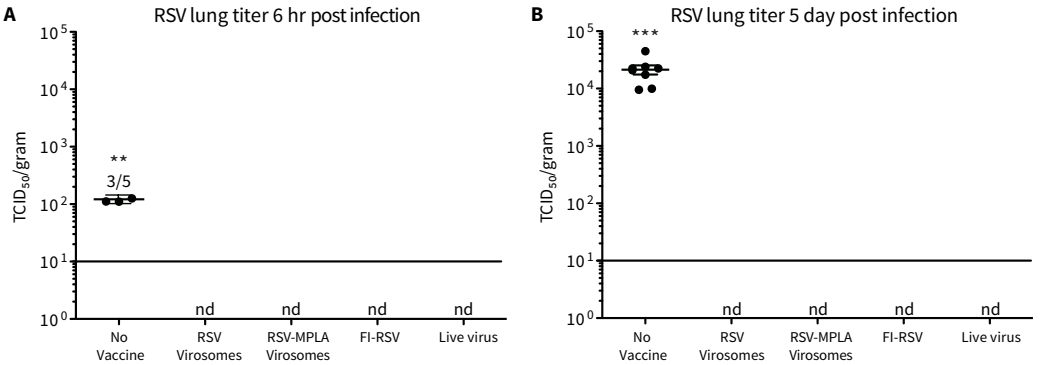


Figure 4.2. In vivo virus clearance after challenge. Cotton rats were challenged with live RSV at day 49. A) 5 rats per group were sacrificed at 6 hours post challenge and lung virus titers were determined by TCID50. B) 7 rats per group were sacrificed at 5 days post challenge and lung virus titers were determined by $TCID_{50}$. Values below 10 $TCID_{50}$ were not detectable. (Mann-Whitney U test **p<0.01, ***p<0.001)

Local immune responses upon RSV infection

To analyze whether RSV-MPLA virosomes prime for immune responses associated with ERD, cotton rats were vaccinated and challenged as described above and cytokine gene expression levels in the lungs of the animals were assessed by qPCR (Figure 4.3 and 4.4).

At 6 h after infection, cotton rats immunized with FI-RSV showed a significant increase of Th2-associated cytokines IL-4, IL-5, IL-9 and IL-13 mRNA expression (Figure 4.3). At this time point, rats immunized with RSV virosomes showed increased levels of IL-5 mRNA as well. In contrast, animals immunized with RSV-MPLA virosomes or live virus did not show significant increases in expression of Th2-associated cytokines at this early time point. At 5 days post infection, IL-4, IL-5 and IL-13 expression remained high in animals immunized with FI-RSV. In contrast, animals immunized with RSV virosomes, RSV-MPLA virosomes or live virus only showed background mRNA levels of these cytokines.

Animals immunized with FI-RSV also showed a significant increase in the Th1-associated cytokine IL-1b, but not IL-12 or IFN-γ mRNA (Figure 4.4). Animals immunized with live virus showed early increases in expression levels of IL-1b, IL-12 and IFN-γ mRNA while non-immunized animals had increased expression levels of IL-1b and IL-12, but not IFN-γ mRNA at 6 h post infection. The levels of IL-12 and IFN-γ mRNA were significantly higher in animals immunized with live virus, than in animals immunized with RSV virosomes, but similar to levels in animals immunized with RSV-MPLA virosomes. At 5 days post infection all the Th1-type cytokine expression levels in the immunized rats had returned to background levels, i.e. levels similar to non-immunized, non-infected animals. Non-immunized animals, however, showed increased levels of IL-12 and IFN-γ at 5 days post-infection.

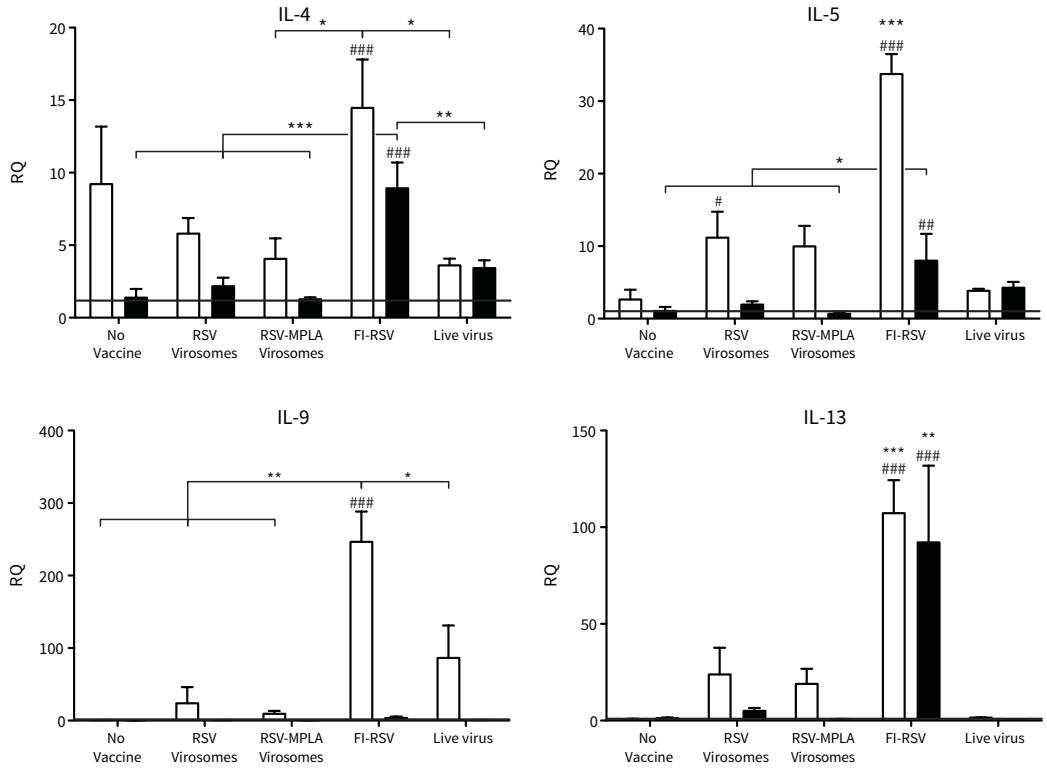


Figure 4.3. Th2-type cytokine expression in the lungs of vaccinated and challenged animals. Cotton rats were vaccinated and challenged as in figure 2 and at 6 hrs (open bars) and 5 days (solid bars) post infection, lung cytokine expression was determined by qPCR. Values are relative quantities (RQ) compared to naïve rats (horizontal line at RQ=1). (Mann-Whitney U test *p<0.5, **p<0.01, ***p<0.001 comparing vaccine group to vaccine group. #p<0.5, ###P<0.001 comparing expression values of the vaccinated and challenged rat to expression in naïve rats.)

Immunopathology upon RSV infection

To assess whether RSV-MPLA virosomes primed for the induction of lung pathology upon infection, we examined the lungs of immunized and challenged animals by means of histology. The lungs of non-immunized animals showed no signs of alveolitis, and the peribronchial and perivascular regions were also free of infiltrates (Figure 4.5A, B). In contrast, the lungs of rats immunized with FI-RSV showed prominent alveolitis as well as peribronchial and perivascular infiltrates (Figure 4.5C, 4.5D). The lungs of animals immunized with RSV virosomes also showed some signs of alveolitis and infiltrates in the peribronchial and perivascular regions (Figure 4.5E, F). In contrast, rats immunized with RSV-MPLA virosomes showed no sign of alveolitis or infiltrates (Figure 4.5G, H) and were comparable to non-immunized animals or animals that received live virus immunization (Figure 4.5I, J).

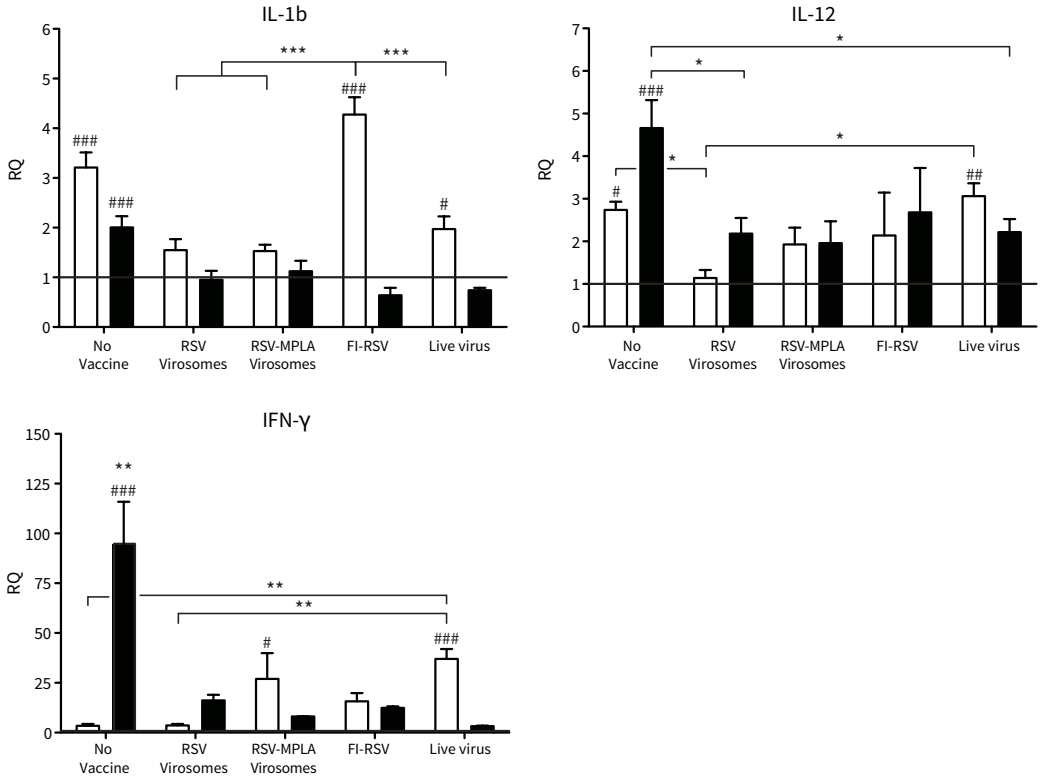
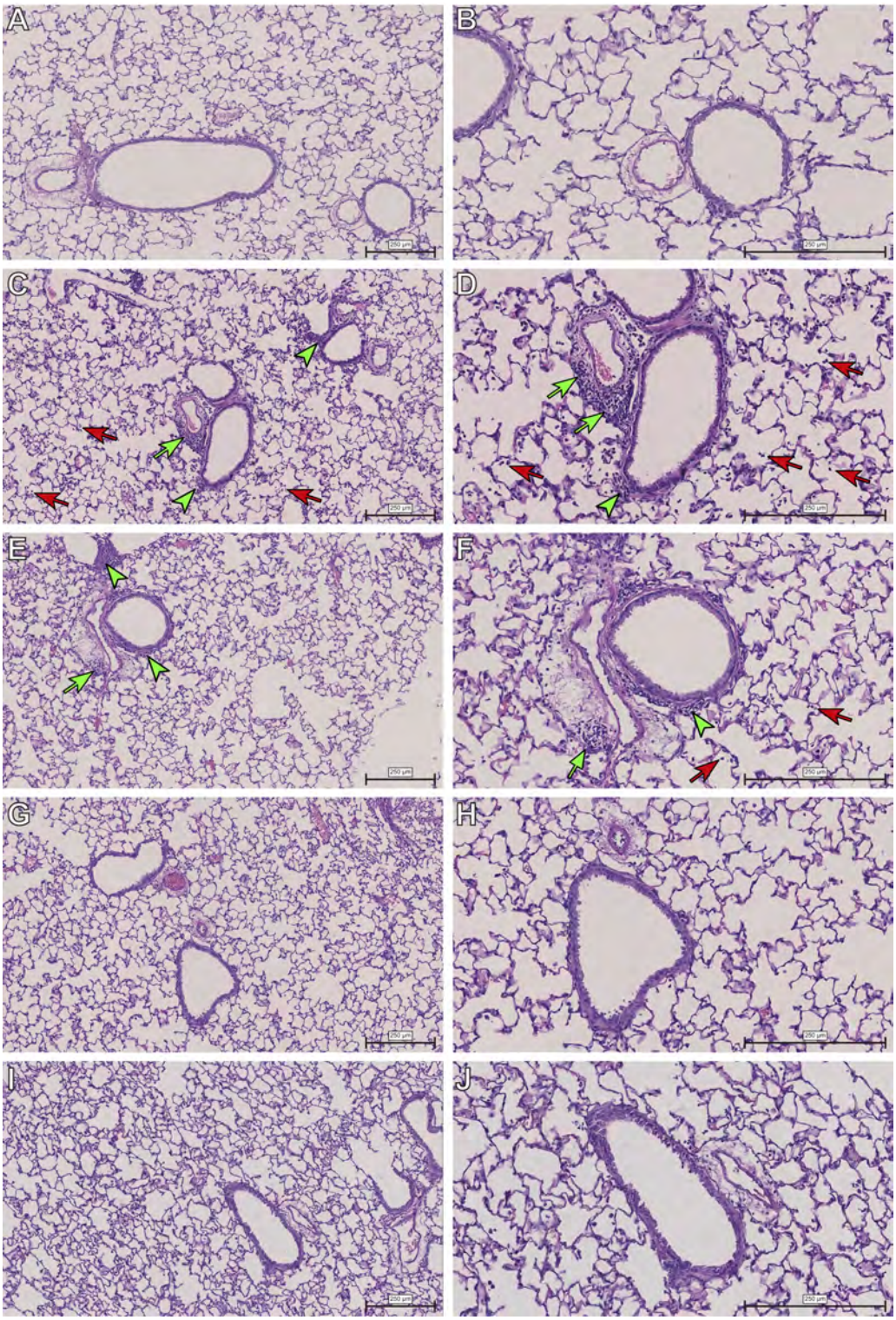


Figure 4.4. Th1-type cytokine expression in the lungs of vaccinated and challenged animals. As figure 4.3 but for Th1-type cytokines. Open bars represent relative transcription abundance at 6hrs post challenge and solid bars at 5 days post challenge.

Finally, infiltrating neutrophils in the alveoli, a hallmark of ERD in both cotton rat and humans,[183] were enumerated. The lungs of rats immunized with RSV-MPLA virosomes contained low numbers of infiltrates similar to those seen in non-immunized animals or animals immunized with live virus. Although we observed an increased number of infiltrating neutrophils in the lungs of the rats immunized with RSV virosomes, the numbers were significantly lower than the numbers induced by immunization with FI-RSV (Figure 4.6).

Figure 4.5 (Opposite page). Cotton rat lung pathology. Cotton rats were vaccinated and challenged as in figure 4.2. At 5 days post challenge lungs were removed, fixed in formalin under 20cm water pressure to preserve the structure of the lungs, embedded in paraffin and stained with H&E. A, B) No vaccine. C,D) FI-RSV. E, F) RSV virosomes. G, H, RSV-MPLA virosomes. I,J) Live virus. Green arrows, perivascular infiltrates. Green arrowheads, peribronchial infiltrates. Red arrows, alveolar infiltrates.


A
B
C
D
E
F
G
H
I
J

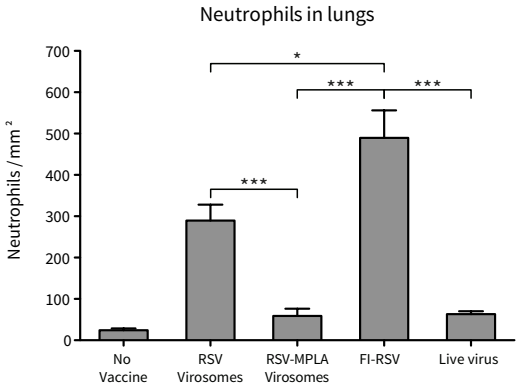


Figure 4.6. Leukocytic alveolar infiltrations. Lungs were treated as in figure 4.5 and the numbers of neutrophils per mm^2 were determined by light microscopy in 4 areas per slide and 3 animals per group. (Mann-Whitney U test *p<0.5, **p<0.01, ***p<0.001)

Discussion

The induction of ERD by FI-RSV in the 1960s clinical trial followed by observations that also other, non-adjuvanted, inactivated vaccine formulations can prime for Th2-type responses and ERD upon RSV infection has tempered enthusiasm for further development of these types of vaccines.[84] Recent insights in the importance of TLR signaling in immunomodulation suggest that addition of TLR ligands to vaccines represents a powerful approach to steer immune responses towards the desired phenotype and have revived the development of inactivated RSV vaccines. Here, we show that a non-replicating virosomal RSV vaccine, with incorporated MPLA, induces virus-neutralizing immune responses without priming for excess Th2-type responses or ERD in RSV–permissive and ERD-prone cotton rats.

In cotton rats immunized with FI-RSV and subsequently challenged with live virus, we observed a strongly upregulated expression of Th2 cytokines including IL-4, IL-5, IL-9 and IL-13. Studies in mice have shown that expression of these cytokines in lungs is implicated in allergen-induced asthma, rhinitis and anaphylaxis.[188-191] IL-5, for example, directly activates eosinophils and is, therefore, an important factor in allergic reactions. IL-9 induces IL-13 expression in the lungs, affecting airway epithelium and contributing to signs of lung pathology.[192] Furthermore, IL-4 and IL-13 upregulate vascular cell adhesion molecule-1 (VCAM-1), which induces migration of eosinophils and lymphocytes. Additionally, upregulation of IL-13 induces mucus secretion in the lungs.[193] Therefore, it appears that expression of Th2 cytokines in the lungs of cotton rats, immunized with FI-RSV and subsequently challenged with live virus, contributes crucially to the observed alveolar, perivascular and peribronchial infiltrations.

The exact mechanism by which FI-RSV skews the immune response to a Th2 -henotype is unknown and most likely multifactorial. For example, antigen alteration by formalin,[80] the presence of cell culture proteins[81] and alum.[82,83] have all been shown to contribute to Th2-associated cytokine expression and ERD. RSV virosomes, on the other hand, are generated from untreated, purified virus, do not contain alum as an adjuvant and, therefore, do not induce excessive Th2 cytokine expression.

Our results show that incorporation of MPLA in the RSV virosomal membrane results in the induction of a balanced Th1/Th2 response. RSV-MPLA virosomes only stimulated the expression of the Th1-associated cytokine IFN-γ, but not of Il-1b or IL-12. In contrast, priming with live virus resulted in expression of all of these three Th1 cytokines after infection, reflecting a more Th1-skewed response than the response induced by RSV-MPLA virosomes. In a previous study in Th2-prone BALB/c mice, we also observed induction of a balanced Th1/Th2 response by RSV-MPLA virosomes.[185] In the latter study, upon immunization of the animals with RSV-MPLA virosomes, we observed higher levels of virus-neutralizing, Th1-signature, IgG2a antibodies and IFN-γ expression, compared to the corresponding responses in mice immunized with FI-RSV. However, similar to the observations made in the present study, the Th1-skewing effect

was more pronounced after priming with live virus than after immunization with RSV-MPLA virosomes. This is in agreement with the findings of Boukhvalova *et al*, who added MPLA to FI-RSV and, after immunization and subsequent challenge in cotton rats, did not observe a strong Th1-skewed response either.[78] They did, however, note a dampening of the pronounced Th2-type response induced by FI-RSV alone.

Comparison of the results obtained with RSV virosomes vs. RSV-MPLA virosomes demonstrates that MPLA is essential for complete prevention of ERD. Even though the expression of Th2 cytokines in cotton rats immunized with non-adjuvanted RSV virosomes was much lower than that in FI-RSV-immunized animals, these animals did display signs of lung pathology. It is possible that these neutrophils were attracted through complement activation, which may have occurred as a result of induction of antibodies with low affinity and low neutralizing capacity (Figure 1), leading to the deposition of immune complexes in the lungs. This may lead to complement fixation, activation and chemo-attraction of neutrophils by complement factor fragments such as C3a, C4a and C5a.[194,195] C3a may additionally induce bronchoconstriction, another symptom of ERD.[68,196] Importantly, immune complex deposition has been demonstrated to play an important role in ERD in mice and immune complex-dependent C4d deposition has been found in lung biopsies from the children who died of ERD in the 1960s trial.[74] Unfortunately, there are, to our knowledge, currently no reagents available to directly investigate immune complex formation and complement fixation in cotton rats.

As indicated before, FI-RSV induces low-affinity antibodies that have poor virus-neutralizing capacity.[72,76] Accordingly, our data indicate that not only FI-RSV, but also non-adjuvanted RSV virosomes, induce inferior neutralizing antibody levels compared to RSV-MPLA virosomes or live virus, pointing to a suboptimal antibody affinity maturation induced by FI-RSV or non-adjuvanted RSV virosomes. In this respect, TLR4 activation by MPLA in RSV-MPLA virosomes or, for that matter, TLR3 activation by RNA in replicative virus are likely to induce proper antibody affinity maturation yielding antibodies more capable of virus neutralization. In the context of inactivated RSV vaccines, it therefore appears that proper TLR activation is essential and sufficient to ensure prevention of ERD upon viral challenge.

Apart from stimulating the induction of high levels of high-affinity antibodies, TLR signaling also improves persistence of the antibody response.[197] Previous reports[183] and our data (Figure 2) show that immunization with FI-RSV gives rise to an antibody response capable of virus neutralization *in vivo,* despite suboptimal affinity maturation of these antibodies. This is most likely due to the high abundance of these antibodies. However, the lack of TLR signaling upon immunization with FI-RSV may cause these antibodies to wane more rapidly than antibodies induced by RSV-MPLA virosomes. This in turn would result soon in titers below those capable of virus neutralization. Thus, in comparison with FI-RSV, RSV-MPLA virosomes, through their ability to activate TLRs, may not only induce better antibody affinity maturation, but also a

more sustained high level of antibodies providing protection against RSV infection for a longer period of time.

Taken together, our results show that RSV-MPLA virosomes induce high levels of virus-neutralizing antibodies, which are able to confer protection from RSV infection in cotton rats. Immunization with RSV-MPLA virosomes does not lead to increased expression of Th2-associated cytokines implicated in the induction of ERD. These data demonstrate the efficacy and safety of RSV-MPLA virosomes as a possible RSV vaccine candidate and warrant further evaluation in clinical trials.

Materials and methods

Ethical statement

Animal experiments were approved by the Committee for Animal Experimentation (DEC) of the University Medical Center Groningen, according to the Dutch Animal Protection Act (permit number DEC 5239D). Immunizations and challenges were conducted under isoflurane anesthesia, and every effort was made to minimize suffering of the animals.

Cells and virus

RSV strain A2 (ATCC VR1540) was kindly donated by Mymetics BV (Leiden, The Netherlands). The virus was grown in HEp-2 cells (ATCC, CL-23, Wesel Germany) in roller bottles in HEp-2 medium: DMEM (Invitrogen, Breda, The Netherlands) supplemented with Pen/Strep, L-Glutamine, Sodium bicarbonate, HEPES, Sodium Pyruvate, 1X Non Essential Amino Acids (all from Invitrogen, Breda, The Netherlands) and 2% FBS (Lonza-Biowhittaker, Basel, Switzerland) and purified on sucrose gradients as described before.[185]

Vaccine formulations

RSV virosomes were generated as described previously.[168] Briefly, the RSV membrane was dissolved in 100 mM 1,2 dihexanoyl-*sn*-glycero-3-phosphocholine (DCPC) in HNE and the nucleocapsid was removed by ultracentrifugation. The supernatant was applied to a dried film of a 2:1 mixture of egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL, USA) at a ratio of 850 nmol lipid per mg of supernatant protein. For incorporation of MPLA, monophosphoryl lipid A from *Salmonella minnesota* Re 595 (Invivogen, Toulouse, France) dissolved in 100 mM DCPC in HNE was added to the protein lipid mixture at 1 mg MPLA per mg supernatant protein, incubated for 15 min at 4°C, filtered through a 0.1 µm filter and dialyzed in a sterile Slide-A-lyzer (10 kD cut-off; Thermo Scientific, Geel, Belgium) against HNE buffer pH 7.4. After dialysis, virosomes were kept at 4°C.

FI-RSV was produced as reported before.[183] FI-RSV was diluted with HNE to contain 5 µg of RSV protein in a 50 µl volume.

Animals and immunizations

Female outbred cotton rats (Hsd:Cotton Rat) of 4-6 weeks old were obtained from Harlan (Indianapolis, IN, USA). Rats received 50 µl RSV virosomes or RSV-MPLA virosomes intramuscularly containing 5 µg of protein. Control rats received 100 µl (10^6 $TCID_{50}$) intranasally, 50 µl of HNE intramuscularly or 50 µl (5 µg viral protein) of FI-RSV intramuscularly. Vaccinations were given on day 0 and day 21. On day 49, rats were challenged with 10^6 $TCID_{50}$ RSV intranasally. At the time of immunization and challenge, blood was drawn by retro-orbital puncture. Six hours or 5 days after challenge, rats were sacrificed and blood was drawn by heart puncture. Lungs were removed aseptically and one of the primary bronchi was ligated just below the tracheal bifurcation with suture wire. Approximately 20 mg of this lobe was removed and stored in 1 ml

of RNAlater (Qiagen, Venlo, The Netherlands) at -20°C for RNA isolation. The remainder this lobe was kept on ice in HEp2 medium containing 2% FBS, for virus titration. The other half of the lung was fixed in 4% formaldehyde in PBS under 20 cm of water pressure to preserve the structure of the lungs for lung histopathology analyses. Control rats used for RNA expression analyses were sacrificed as described above without prior challenge.

IgG antibody ELISA

RSV-specific serum IgG titers were determined using standard protocols[168] using horseradish-peroxidase-coupled goat anti-mouse IgG (Southern Biotech 1030-05) which was cross reactive for cotton rat IgG.

Virus titration and microneutralization assay

Virus titers were determined by $TCID_{50}$ as described previously.[185] RSV virus neutralization titers were determined by incubation of two-fold serially diluted cotton rat serum with 70 $TCID_{50}$ of RSV for 2 h and subsequent titration of this mixture on HEp-2 cells as described before.[185] The neutralization titer was calculated with the Reed & Muench method as the dilution that neutralizes infection in 50% of the wells.

Lung histopathology

The inflated lungs were embedded in paraffin and 4 µm slices were cut. The slides were then stained with hematoxylin and eosin (H&E) using standard procedures. Subsequently, perivasculitis, peribronchiolitis and alveolitis were assessed by light-microscopy. Cellular infiltrates (neutrophils) in the alveoli were assessed based on morphology.

Lung RNA isolation

Lung samples stored in RNA later were transferred to tubes containing 300 µl RLT buffer (Qiagen, Venlo, The Netherlands) and homogenized using disposable pestles with a pestle motor (VWR, Amsterdam, The Netherlands). Subsequently RNA was isolated with an RNeasy mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's protocol. After isolation RNA concentration was determined on a Nanodrop (Thermo scientific, Wilmington, DE, USA).

cDNA synthesis and qPCR

cDNA synthesis and qPCR were performed using the Verso cDNA synthesis kit (Acros Organics, Geel, Belgium) and Absolute QPCR SYBR Green Mix (Westburg, Leusden, The Netherlands) according to the manufacturer's protocols. Cotton rat cytokine sequences were obtained from NCBI[187] and used to generate primers that yield products of approximately 125 bp (table 4.1). PCR analyses were run on an Applied Biosystems AB7500 Real-Time PCR system (Applied Biosystems, Nieuwerkerk ad IJssel, The Netherlands) with the following conditions: 15 min 95°C, 40 times 30 sec 95°C, 30 sec 60°C followed by a melt curve. Relative transcript abundance was calculated using the 2^{-ddCt} method comparing Ct (cycle threshold) values from the immunized and challenged rats to non-immunized, non-challenged rats corrected for Ct values for the

housekeeping gene β-actin.

Table 4.1. Primers for cotton rat cytokine RT-PCR

Th1	Sense primer	Antisense primer	Product length
IL-1b	GTGCTGTCTGACCCATGTGAGC	GCCACTGGTATCTTGGTGCTGC	122
IFN-y	CATCAACACCATCAGGGCGGAC	TGACAGCTTTGCGCTGGACC	125
IL-12-p40	CCACTCACGTCTGCTGCTTCAC	ACCGGCCAGAGTAATTTGCTGC	125
Th2			
IL-4	GCTTCCCAGGTGCTTCGCAAG	TGGAGACAGACTGCTGATGCCC	120
IL-5	GGGGCACTGTGGAAACGCTATTC	GCAGGTAATCCAGGAACTGCCG	117
IL-9	TGTGACCAGTTGCTTGTGTCTTCC	ACCCGATTGAAAAGAACCGAGAACC	126
IL-13	TGCCTCGATGCATGTCTCCTCC	GCTGTCAGGTCCACGCTCCATAC	124
Housekeeping gene			
β-actin	CCCCAAGGCCAACCGTGAAAAG	CACAATGCCAGTGGTACGACCAG	124

Statistical analyses

All statistical analyses were performed with Graphad Prism 5.00 for Mac OSX, (GraphPad Software, San Diego California USA, www.graphpad.com). Statistical significance was assessed using a Mann-Whitney U test. P values of 0.05 or lower were considered to represent significant differences.

Chapter 5

Efficacy and safety of an intranasal virosomal Respiratory Syncytial Virus vaccine adjuvanted with Monophosphoryl Lipid A in mice and cotton rats

Tobias Kamphuis[1], Muhammad Shafique[1], Tjarko Meijerhof[1], Toon Stegmann[1,2], Jan Wilschut[1], Aalzen de Haan[1]

1) Department of Medical Microbiology, Molecular Virology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
2) Mymetics BV Leiden, The Netherlands

Submitted to Vaccine

Abstract

Respiratory syncytial virus infection remains a serious health problem, not only in infants but also in immunocompromised adults and the elderly. An effective and safe vaccine is not available due to several obstacles: non-replicating RSV vaccines may prime for excess Th2-type responses and enhanced respiratory disease (ERD) upon natural RSV infection of vaccine recipients. We previously found that inclusion of the Toll-like receptor 4 (TLR4) ligand monophosphoryl lipid A (MPLA) in reconstituted RSV membranes (virosomes) potentiates vaccine-induced immunity and skews immune responses toward a Th1-phenotype, without priming for ERD. As mucosal immunization is an attractive approach for induction of RSV-specific systemic and mucosal antibody responses and TLR ligands could potentiate such responses, we explored the efficacy and safety of RSV-MPLA virosomes administered intranasally (IN) to mice and cotton rats.

In mice, we found that incorporation of MPLA in IN-administered RSV virosomes increased both systemic IgG and local secretory-IgA (S-IgA) antibody levels, increased Th1-type skewing and resulted in significantly reduced lung viral titers upon live virus challenge. Antibody responses and Th1/Th2-cytokine responses induced by RSV-MPLA virosomes were comparable to those induced by live RSV infection. By comparison, formalin-inactivated RSV (FI-RSV) induced serum IgG that inhibited viral shedding upon challenge, but also induced Th2-skewed responses. In cotton rats, similar effects of incorporation of MPLA in virosomes were observed with respect to induction of systemic antibodies and inhibition of lung viral shedding upon challenge, but mucosal sS-IgA responses were only moderately enhanced. Importantly, IN immunization with RSV-MPLA virosomes, like live virus infection, did not lead to any signs of ERD upon live virus challenge of vaccinated animals, whereas IM immunization with FI-RSV did induce severe lung immunopathology under otherwise comparable conditions.

Taken together, these data show that mucosally administered RSV-MPLA virosomes hold promise for induction of safe and protective immunity against RSV.

Introduction

Respiratory syncytial virus (RSV) infection causes viral bronchiolitis in infants and young children but also significant health problem in the elderly and immune-compromised individuals.[1,7,8] RSV infection at young age does not lead to life-long protection and multiple reinfections occur throughout life.[6,7,164] Vaccination of risk groups would be an effective approach to reduce the burden of disease. Although RSV has been recognized as an important vaccine target, no vaccine is available. This is, in part, due to the fact that immunization with inactivated RSV formulations or purified protein preparations can prime for enhanced respiratory disease (ERD) upon natural infection,[144] as did a formalin-inactivated RSV vaccine (FI-RSV), evaluated in young children in the 1960s.[68-70] Hallmarks of ERD are neutrophilic alveolar infiltrates as well as perivascular and peribronchial infiltration of lymphocytes.[183] Immunization with FI-RSV also led to the induction of poorly neutralizing antibodies[72,73] as a result of impaired affinity maturation, probably because of a lack of Toll-like Receptor (TLR) signaling by FI-RSV.[76] Subsequent work in animal models showed that FI-RSV also induces Th2-skewed immune responses, as opposed to Th1-type responses that are better suited to protect against viral infections.[77] An approach to induce better neutralizing antibodies and Th1-skewed responses and to avoid priming for ERD, is to incorporate TLR ligands as immunomodulators in candidate non-replicating RSV vaccines.[78]

RSV enters through the mucosal surface of the respiratory tract. A desirable feature of RSV vaccines would therefore be the capacity to induce, besides systemic antibody responses, also local immunity against RSV like secretory IgA antibodies (S-IgA). Mucosal immunization, through intranasal (IN) administration, could achieve such responses. It not only is a non-invasive and highly acceptable route of administration,[198] in addition, it does not readily prime for enhanced disease, at least in animal models.[199] However, as mucosal surfaces are continuously exposed to antigens, mucosal immune tolerance mechanisms prevent untoward immune reactions. Therefore, inclusion of TLR ligands in an IN-administered RSV vaccine may well represent an essential prerequisite for induction of robust RSV-specific mucosal as well as systemic antibody responses.[200]

A TLR ligand currently used in two registered intramuscular (IM) human vaccines is monophosphoryl lipid A (MPLA).[127,128] We found that MPLA in RSV virosomes induces safe and protective immune responses in mice and cotton rats upon IM injection.[185] Interestingly, MPLA has also been reported to have adjuvant activity when co-administered IN with different vaccine antigens.[201,202] These findings therefore prompted further exploration of our candidate MPLA-adjuvanted RSV virosomal vaccine for induction of RSV-specific immunity upon IN administration.

Incorporation of MPLA in RSV virosomes administered IN to mice potentiated protective RSV-specific serum IgG and respiratory tract S-IgA antibody responses and induced Th1-skewed T-cell responses. Incorporation of MPLA in RSV virosomes administered IN to cotton rats

significantly increased virus-neutralizing serum IgG responses and protection against infection but only moderately stimulated mucosal S-IgA responses. In contrast to IM injection of FI-RSV, IN administration of RSV-MPLA virosomes did not prime for lung immunopathology upon challenge. These data combined show that mucosally administered RSV-MPLA virosomes hold promise for induction of protective immunity without priming for enhanced disease.

Results

Immunogenicity in mice

To determine the adjuvant effect of MPLA in IN-administered RSV virosomes, we immunized mice with RSV virosomes or RSV-MPLA virosomes. RSV-naïve mice and mice immunized with FI-RSV or live virus, served as controls. RSV-MPLA virosomes, but not virosomes without MPLA, induced RSV-specific serum IgG, although levels were significantly lower than those induced by IM injection with FI-RSV or live RSV infection (Figure 5.1A). One of six mice receiving a second IN immunization with RSV virosomes developed detectable RSV-specific serum IgG antibodies. In contrast, all mice that received a second IN immunization with RSV-MPLA virosomes developed IgG antibodies, to similar levels as in mice that received a second immunization with FI-RSV or a live virus infection (Figure 5.1A).

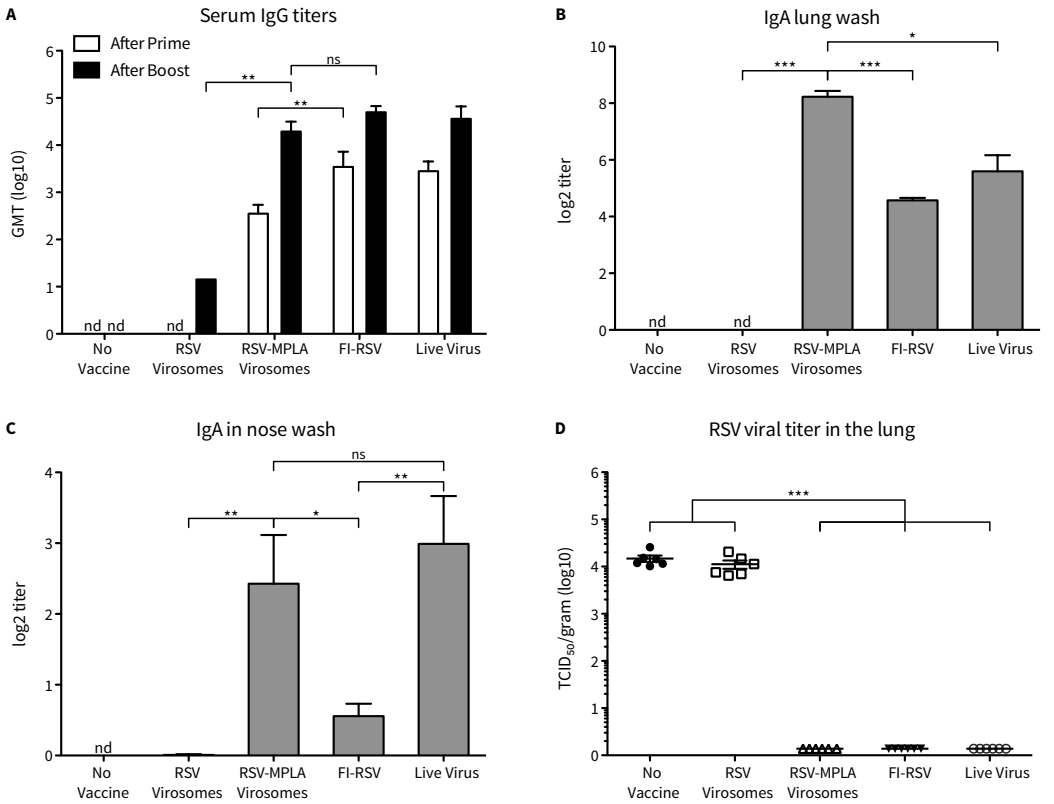


Figure 5.1. Immunogenicity and protection in mice. Mice were immunized twice ("prime" on day 0 and "boost" on day 14) with RSV virosomes IN, RSV-MPLA virosomes IN, FI-RSV IM, and live virus IN. Control mice received buffer IN. A) Fourteen days after the immunizations blood was drawn and RSV-specific IgG in serum was determined. The immunized mice were challenged on day 28 with 106 $TCID_{50}$ RSV and terminated 5 days later. B, C) After termination IgA titers were determined in lung wash and nose wash. D) RSV virus titers were determined by $TCID_{50}$ on lung homogenates. (Mann-Whitney U test: ns not significant,*p<0.05, **p<0.01, ***p<0.001)

For assessment of local immune responses, we analyzed lung and nose wash RSV-specific IgA antibodies. Mice immunized twice IN with RSV-MPLA virosomes showed significantly higher S-IgA levels in lungs compared to mice immunized with non-adjuvanted virosomes, FI-RSV or live virus infection. Both RSV-MPLA virosomes and live virus infection induced significantly higher nasal S-IgA compared to levels induced by non-adjuvanted virosomes and FI-RSV (Figure 5.1B,C).

To determine protection against infection, immunized mice were infected with live RSV. Non-vaccinated mice or mice immunized IN with non-adjuvanted virosomes showed virus titers of approximately 10^4 $TCID_{50}$, 4 days post-infection. On the other hand, animals immunized with RSV-MPLA virosomes, FI-RSV or live virus had no detectable lung virus titers (Figure 5.1D).

Cellular immune response

To determine if the induced immune responses had Th1- or Th2-skewed phenotypes, we determined IFN-γ and IL-5 levels in RSV-restimulated splenocytes from immunized and subsequently challenged animals of all groups (Figure 5.2). IFN-γ production in splenocyte cultures from mice immunized with RSV-MPLA virosomes was significantly higher than that in cultures from mice immunized with non-adjuvanted virosomes or FI-RSV. In contrast, IL-5 production in splenocyte cultures from mice immunized with FI-RSV was significantly higher than that in cultures from mice immunized with non-adjuvanted RSV virosomes, RSV-MPLA virosomes or live virus infection. Therefore, RSV-MPLA virosomes and live virus infection induced Th1-skewed responses while FI-RSV induced clear Th2-skewed responses.

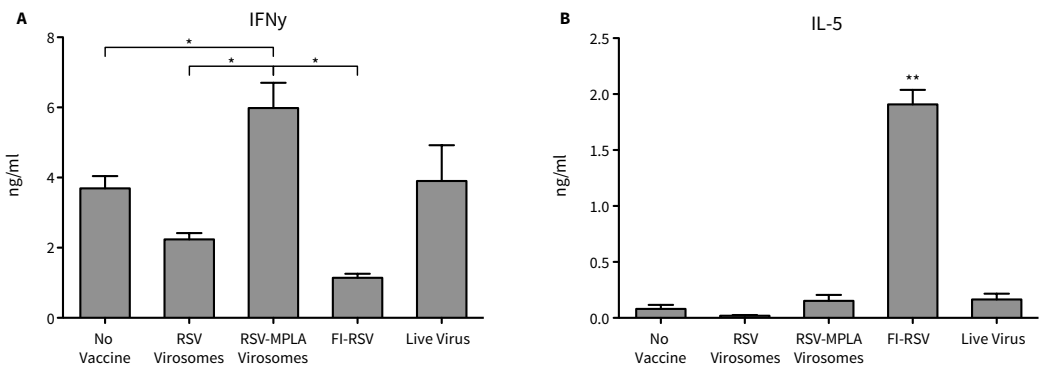


Figure 5.2. Cellular immune response in mice. Mice were immunized, challenged and terminated as in Figure 1. After termination, spleens were harvested and splenocytes were restimulated in vitro with BPL-inactivated RSV for three days. After three days IFN-γ (A) and IL-5 (B) were determined in the supernatants. (Mann-Whitney U test:,*p<0.05, ***p<0.001).

Immunogenicity in cotton rats

Next, we evaluated immune responses, protection and vaccine-induced immunopathology in the cotton rat model. Cotton rats, compared to mice, are more permissive to RSV and more prone to develop ERD. Similar to antibody responses in mice, low levels of RSV-specific serum IgG were detected in cotton rats immunized IN with non-adjuvanted RSV virosomes (Figure 3A). However, incorporation of MPLA in the IN-administered virosomes significantly increased systemic IgG levels. Animals immunized once with RSV-MPLA virosomes showed significantly lower titers compared to those in cotton rats immunized once with FI-RSV or live virus. However, serum IgG antibody levels increased after the booster immunization to similar levels as seen in cotton rats primed and boosted with FI-RSV or live virus (Figure 5.3A).

Next, the virus-neutralizing capacity of the sera were assessed. Sera from RSV-naïve cotton rats or rats immunized IN with non-adjuvanted RSV virosomes, did not have any significant neutralizing capacity (Figure 5.3B). RSV-MPLA virosomes induced significantly increased levels of neutralizing antibodies compared to RSV virosomes without MPLA. These levels were, on average, also higher than those induced by IM immunization with FI-RSV, although the difference did not reach statistical significance. Live RSV infection, however, induced significantly higher neutralizing antibody levels compared to those induced by RSV-MPLA virosomes, administered IN, or FI-RSV, injected IM.

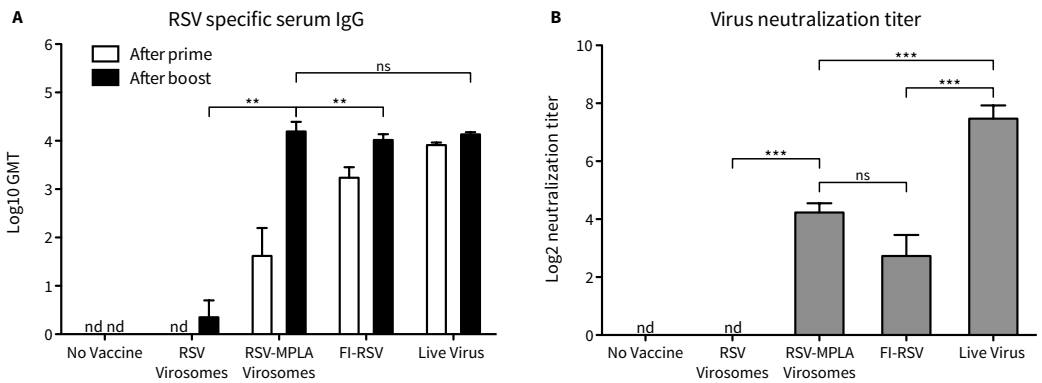


Figure 5.3. Immunogenicity in cotton rats. Cotton rats were immunized with the same preparations as given to mice (Figure 1) on day 0 and 21. A) On day 21 and 49, blood was taken en RSV specific IgG was determined in serum. B) RSV-virus neutralizing antibodies were determined in the day 49 serum. (Mann-Whitney U test: ns not significant,*p<0.05, **p<0.01, ***p<0.001).

Finally, the local antibody responses were determined. Although MPLA increased nasal S-IgA levels, levels of S-IgA in nose and lung induced by live virus infection were significantly higher than those observed in the other groups (Figure 5.4)

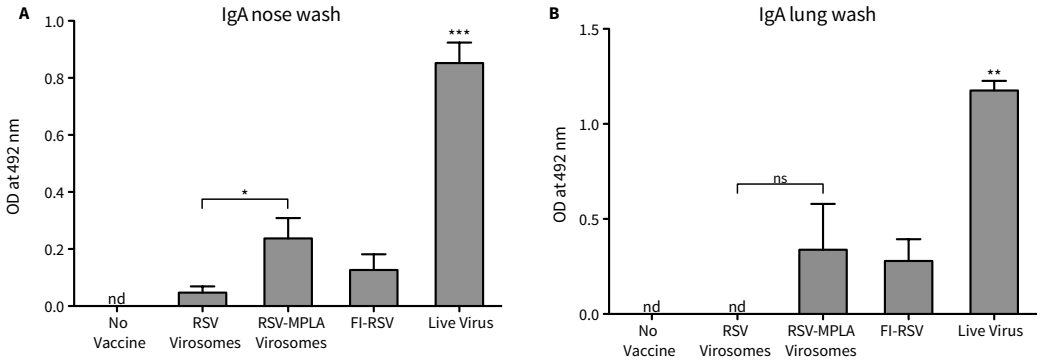


Figure 5.4. Mucosal immune response in cotton rats. Cotton rats were immunized as in Figure 5.3 and challenged with 106 TCID50 live RSV on day 49. Five days after challenge, the rats were sacrificed and lung and nose washes were taken. A, B) RSV specific IgA was determined in nose washes and lung washes. (Mann-Whitney U test:*p<0.05, **p<0.01, ***p<0.001)

Protection from RSV challenge

To determine protection against infection, immunized cotton rats were infected with live RSV. Significant lung virus titers were detected in RSV-naïve cotton rats and cotton rats immunized IN with non-adjuvanted RSV virosomes (Figure 5.5). Five out of seven cotton rats immunized IN with RSV-MPLA virosomes showed no lung virus titers, while two cotton rats had detectable virus titers, but at levels that were significantly lower compared to those in animals immunized IN with non-adjuvanted RSV virosomes. All animals immunized with FI-RSV or live virus infection had non-detectable lung viral shedding (Figure 5.5).

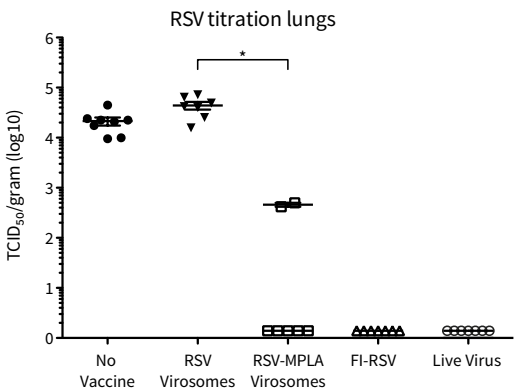
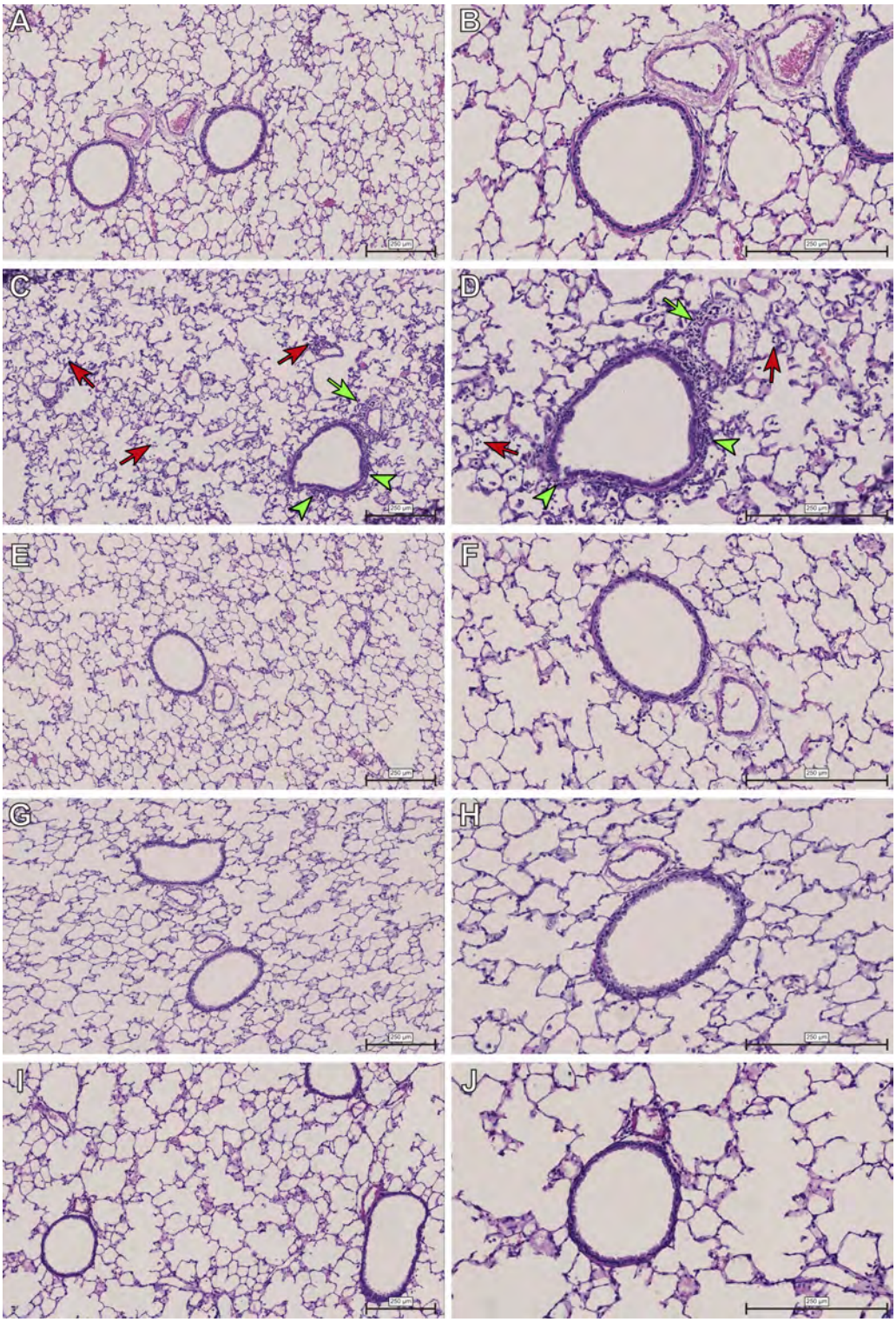


Figure 5.5. Protection in cotton rats. Cotton rats were immunized, challenged and sacrificed as in Figure 5.4. After termination, the lungs were removed and RSV titers in lungs were determined by $TCID_{50}$. (Mann-Whitney U test: ns not significant,*p<0.05, **p<0.01, ***p<0.001)

Figure 5.6. (Opposite page) Lung pathology in cotton rats. Cotton rats were immunized, challenged and sacrificed as in Figure 5.4. After termination, one lung lobe was fixated with 4% formalin under 20 cm water pressure to retain the structure of the lung. After fixation, the lungs were embedded in paraffin and 4 µm slices were cut and stained with H&E. The lungs were evaluated by light microscopy. Groups: A,B) No vaccine, C,D) FI-RSV, E,F) RSV virosomes, G,H) RSV-MPLA virosomes, I,J) Live virus. Red arrows, alveolar infiltrates, green arrows, perivascular infiltrates, green arrowheads, peribronchial infiltrates.


A
B
C
D
E
F
G
H
I
J

Histopathology analyses

To evaluate the safety of IN administration of RSV virosomes and RSV-MPLA virosomes, we harvested the lungs of immunized and challenged cotton rats and assessed lung pathology by light microscopy. Lungs from cotton rats immunized IM with FI-RSV showed clear signs of ERD, with perivascular and peribronchial infiltration and alveolitis with influx of predominantly neutrophils (Figure 5.6 C,D). The lungs of non-immunized animals (Figure 5.6 A,B), animals immunized IN with RSV virosomes (Figure 5.6 E,F), RSV-MPLA virosomes (Figure 5.6 G,H) or live virus (Figure 5.6 I,J), however, did not show signs of ERD.

Discussion

Intranasal administration represents an attractive route of administration for vaccines, including RSV vaccines. Effective induction of immune responses with non-replicating vaccine antigens through this route usually requires the use of adjuvants.[203] The adjuvant MPLA has an acceptable safety profile in humans and is currently being used in a number of licensed vaccines.[115,174] It does not only have immunomodulatory properties for induction of safe Th1-skewed responses against RSV,[78,185] but also has been reported to have mucosal immunoadjuvant properties.[201,202] However, MPLA has not been tested before for its capacity to potentiate immune responses to a non-replicating RSV vaccine, such as RSV virosomes, upon IN administration. Here, we show that RSV virosomes with incorporated MPLA have the capacity to induce protective immune responses upon IN administration to mice and cotton rats, without priming for ERD.

IN administration of RSV-MPLA virosomes effectively induces serum IgG antibody responses and Th1-skewed immune responses, similar to RSV-MPLA virosomes administered by IM injection.[185] This is line with previous findings by others who compared the immunoadjuvant activity of MPLA co-administered IN or parenterally with antigen.[204] The adjuvant effect of MPLA is likely caused by the direct interaction of MPLA with TLR4 on dendritic cells (DC) that are abundantly present in draining lymph nodes, nasal or bronchus-associated lymphoid tissue, or even directly lining the respiratory tract. The activation leads to secretion of IL-12 and type 1 IFN, which skew T cell responses toward a Th1-phenotype.[205] Similar to DC, B cells may be directly activated through TLR4 signaling which, together with aid of the induced T cell response, stimulates antibody responses.[180] Although many cell types in the respiratory mucosa express TLR4, the receptor for MPLA, the expression of the co-receptors CD14 and MD2, which are crucial for the initiation of TLR4-mediated cell signaling, are expressed at a lower level compared to their expression on, for instance, DC.[206] This reduced expression of CD14 and MD2 on the mucosal cell surfaces, e.g. epithelial cells, may reduce their susceptibility to endotoxins but possibly also to stimulatory effects of vaccine adjuvants such as MPLA.[207] This could explain the lower levels of RSV-neutralizing antibodies induced by IN immunization compared to IM immunization.[185] Other TLRs, such as TLR2 and TLR5, do not require these adaptor molecules and are also abundantly expressed on cells in the mucosal surfaces[206] and ligands for these receptors have been reported to have strong mucosal immunoadjuvant properties too.[208,209] How the mucosal immunoadjuvant activity of MPLA, co-administered in RSV virosomes, compares with that of other virosome-incorporated TLR ligands, such as a TLR2 ligand,[168] remains to be investigated further.

Mucosal immunization can induce local S-IgA antibodies. More robust local S-IgA was induced by RSV infection in cotton rats, particularly when compared to responses induced by IN immunization with virosomes. In mice, differences in levels of S-IgA induced by infection or IN immunization were less pronounced. This difference may be related to the much higher permissiveness of the cotton rat for RSV infection than that of mice,[141] leading to higher levels

of viral replication and stronger local immune activation and, consequently, to higher S-IgA responses. Because FI-RSV also induces serum IgA (unpublished results), IgA found in washes of cotton rats or mice immunized with FI-RSV may originate from serum and translocate to the mucosa by transudation (in case of monomeric IgA) or through transcytosis mediated by the polymeric immunoglobulin receptor (pIgR; in case of polymeric IgA).[210] Interestingly, TLR4 signaling upregulates expression of pIgR responsible for polymeric IgA transcytosis.[210,211] Clearly, RSV-specific serum IgG alone, for example induced by IM injection of RSV-MPLA virosomes, inhibits virus shedding in the lung. In this respect, we previously observed that lung viral titers negatively correlate with RSV-specific serum IgG levels,[200] pointing to (sufficient levels of) serum IgG, as an important mediator of protection of the lungs. The upper respiratory tract, however, may not benefit so much from serum IgG for protection against infection, as transudation of antibody to this site is less efficient.[212] Rather, local S-IgA antibody may be more important for protection against viral infection at this site, as has previously been reported for influenza.[213] Further studies should clarify if S-IgA protects the upper respiratory tract by specifically analyzing nasal virus shedding in cotton rats immunized IN.

Together our data show that RSV-MPLA virosomes have the capacity to induce protective immunity upon IN administration to mice and cotton rats, without priming for enhanced disease. IN administration forms an attractive alternative to IM injection, as it is a non-invasive route of administration. Clearly, to potentiate RSV virosome-induced immune responses through this route, adjuvants are needed, which could be MPLA or possibly other TLR ligands with mucosal immunoadjuvant properties.

Materials and Methods

Ethical statement

Animal experiments were approved by the Committee for Animal Experimentation (DEC) of the University Medical Center Groningen, according to the guidelines provided by the Dutch Animal Protection Act (permit number DEC 5239A and 5239D). Immunizations and challenges were conducted under isoflurane anesthesia and every effort was made to minimize suffering of the animals.

Virus

RSV strain A2 (ATCC VR1540) was kindly donated by Mymetics BV (Leiden, The Netherlands). The virus was grown in HEp-2 cells (ATCC, CL-23, Wesel, Germany) and purified as described before.[185]

Vaccine formulations

RSV virosomes were generated as described previously.[168] Briefly, RSV membranes were dissolved in 100 mM 1,2 dihexanoyl-*sn*-glycero-3-phosphocholine (DCPC) in HNE (5 mM Hepes, 145 mM NaCl, 1 mM EDTA, pH 7.4) and the nucleocapsid was removed by ultracentrifugation. The supernatant was applied to a dried film of a 2:1 mixture of egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL, USA) at a ratio of 850 nmol lipid per mg of supernatant protein. For incorporation of MPLA, monophosphoryl lipid A from *Salmonella minnesota* Re 595 (Invivogen, Toulouse, France) dissolved in 100 mM DCPC in HNE was added to the protein lipid mixture at 1 mg MPLA per mg supernatant protein, incubated for 15 min at 4 ˚C, filtered through a 0.1 µm filter and dialyzed in a sterile Slide-A-lyzer (10 kD cut-off; Thermo Scientific, Geel, Belgium) against HNE. After dialysis, virosomes were kept at 4 ˚C. FI-RSV was produced as reported before.[183]

Mouse immunization and challenge experiments

Female BALB/c OlaHsd mice (6-8 weeks old) were purchased from Harlan (Zeist, The Netherlands). For immunization and challenge, mice were anesthetized using isoflurane. Mice received RSV(-MPLA) virosomes (5 µg viral protein) IN in 50 µl HNE. Control mice received 25 µl of FI-RSV (5 µg viral protein) IM, 50 µl (10^6 $TCID_{50}$) of live RSV IN or 50 µl of HNE IN. Vaccinations were given on day 0 and day 14. On day 28 mice were challenged with 10^6 $TCID_{50}$ of live RSV IN. On time points of vaccination and challenge, blood was drawn by retro-orbital puncture. Four days after challenge, mice were sacrificed and blood was sampled. Nose washes and broncho-alveolar lavages were done by incising the trachea and flushing of 1 ml PBS with protease inhibitors (Roche, Mannheim, Germany). Spleens were harvested for analysis of RSV-specific T cell cytokine responses and lungs for analysis of viral titers.

Cotton rat immunization and challenge experiments

Female outbred cotton rats (Hsd:Cotton Rat) of 4-6 weeks old were obtained from Harlan

(Indianapolis, IN, USA). Rats received RSV(-MPLA) virosomes IN (5 µg viral protein). Control rats received 100 µl live virus (10^6 $TCID_{50}$) IN, 100 µl of HNE IN or 50 µl (5 µg viral protein) of FI-RSV IM. Vaccinations were given on day 0 and day 21. On day 49, rats were challenged with 10^6 $TCID_{50}$ RSV IN. At the time of immunization and challenge, blood was drawn by retro-orbital puncture. Five days after challenge, rats were sacrificed and blood was sampled. Lung and nose washes were performed using similar techniques as in mice. Subsequently, the lungs were removed aseptically and one of the primary bronchi was ligated just below the tracheal bifurcation with suture wire. Half of the lung was kept on ice in HEp-2 medium containing 2% FBS, for virus titration. The other half of the lung was fixed in 4% formaldehyde in PBS under 20 cm of water pressure to preserve the structure of the lungs for lung histopathology analyses.

Immunological assays

RSV-specific antibody titers were determined as described before.[168] Briefly, 96-well plates were coated with betapropiolactone-inactivated RSV and blocked with 2.5% milk powder in coating buffer. Plates were incubated for 90 min with two-fold serial dilutions of serum or broncho-alveolar lavages, starting at dilutions of 1:200 for serum or 1:1 for BAL or nose washes. After washing, plates were incubated with a 1:5000 dilution of horseradish-peroxidase-coupled goat anti-mouse IgG, or IgA which bind to both mouse and cotton rat IgG and IgA, respectively (Southern Biotech 1030-05, 1040-05) for 1 h and subsequently stained with *o*-Phenylenediamine (OPD; Sigma-Aldrich, St Louis, MO, USA). After 30 min the staining was stopped by addition of 2 M H_2SO_4 and absorption was measured at 492 nm.

IFN-γ and IL-5 secretion in RSV-stimulated splenocyte cultures were assessed as described before.[185]

Virus titration and microneutralization assay

Virus titers were determined by $TCID_{50}$ as described previously.[185] For determination of RSV virus neutralization titers, serum was decomplemented by heat inactivation for 30 min at 56˚C. Neutralization titers were determined by incubation of two-fold serially diluted decomplemented serum with 70 $TCID_{50}$ of RSV for 2 h and subsequent titration of this mixture on HEp-2 cells as described before.[185] The neutralization titer was calculated with the Reed & Muench method as the dilution that neutralizes infection in 50% of the wells.

Histopathology

The inflated cotton rat lungs were embedded in paraffin and 4 µm slices were cut. The slides were stained with hematoxylin and eosin (H&E) using standard procedures. Subsequently, perivasculitis, peribronchiolitis and alveolitis were assessed by light-microscopy.

Statistical analysis

Statistical analyses were performed with Graphad Prism 5.00 for Mac OSX, (GraphPad Software,

San Diego California USA, www.graphpad.com) using a Mann-Whitney U test. P values of 0.05 or lower were considered to represent significant differences.

Chapter 6

A virosomal Respiratory Syncytial Virus vaccine adjuvanted with Monophosphoryl Lipid A:
Immunogenicity and protective efficacy in aged cotton rats

Tobias Kamphuis[1], Toon Stegmann[1,2], Tjarko Meijerhof[1], Jan Wilschut[1], Aalzen de Haan[1]

1) Department of Medical Microbiology, Molecular Virology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
2) Mymetics BV Leiden, The Netherlands

Manuscript in preparation

Abstract

Each year, respiratory syncytial virus (RSV) infection is responsible for 180,000 hospitalizations and 14,000 deaths among the elderly in the USA alone, causing a disease burden similar to that of influenza in this population. Vaccination could significantly lower morbidity and mortality due to RSV among the elderly. Here, we investigated if a virosomal RSV vaccine has the capacity to induce protective immune responses upon intramuscular (IM) injection in aged cotton rats and studied if adjuvantation of virosomes with monophosphoryl lipid A (MPLA) further stimulates antibody responses. Furthermore, we studied the induction and waning of serum antibody responses induced by a priming infection early in life and the capacity of virosomal RSV vaccine to boost these antibodies in aged cotton rats.

Intramuscular (IM) injection of RSV virosomes induced protective RSV-specific serum IgG antibody responses in aged cotton rats. Immunization with RSV-MPLA virosomes further enhanced serum IgG levels compared to non-adjuvanted virosomes, without priming for enhanced lung pathology. RSV-specific serum antibodies, induced by infection early in life, waned significantly upon aging, but protective levels of specific antibody were still present at old age. RSV-specific serum antibody levels, however, increased after IM injection of RSV virosomes, irrespective of whether the virosomes were adjuvanted with MPLA or not. RSV-specific secretory IgA (S-IgA) in the the respiratory tract of primed and aged cotton rats increased after intranasal (IN) immunization, but not after IM immunization, with virosomal RSV vaccine, and incorporation of MPLA in the virosomes boosted this response.

Taken together, these data show that RSV-MPLA virosomes are immunogenic in an aged immune system and are able to boost preexisting immunity against RSV.

Introduction

Respiratory syncytial virus (RSV) is the major cause of viral bronchiolitis in children and also a significant problem in the elderly. By the age of two, nearly all children have been infected with RSV at least once.[5] RSV infection does not lead to life-long protection and multiple reinfections occur throughout life. Previous studies pointed to RSV-specific serum IgG, but also mucosal secretory IgA (S-IgA), as protective effector mechanisms in RSV infection of adults.[214] In healthy adults with insufficient immunity, reinfections may lead to mild disease with common cold-like symptoms.[6] At older age, however, the immune system weakens and RSV infections can induce severe disease.[65,164] In the elderly, RSV is responsible for approximately 180,000 hospitalizations and 14,000 deaths each year in the USA alone.[7] Compared to influenza, RSV contributes to a similar extent to severe lower respiratory infections in the elderly.[7] Treatment of elderly suffering from RSV is mainly supportive and consist of administration of fluids and in some cases bronchodilatators.[65] A vaccine against RSV, however, could significantly reduce the burden of disease in the elderly.

We previously showed that reconstituted RSV membranes, adjuvanted with the Toll-Like Receptor 4 (TLR4) ligand monophosphoryl lipid A (MPLA) represent a promising vaccine candidate. Studies in mice and cotton rats showed that RSV-MPLA virosomes induce high levels of serum RSV-specific, virus-neutralizing, antibodies and a balanced Th1/Th2 response.[185] Immunization of mice or cotton rats with RSV-MPLA virosomes does not prime for lung pathology (i.e. enhanced respiratory disease[68,77,183]) upon natural infection in contrast to immunization with formalin-inactivated RSV (FI-RSV). It is not known whether the RSV virosomal vaccine has the capacity to potentiate immune responses in an aged immune system and, if MPLA has the capacity to boost responses under these conditions.

A suitable animal model to study the efficacy of RSV vaccines is the cotton rat (*Sigmodon hispidus)*. Cotton rats are more permissive than mice for RSV and show signs enhanced disease lung pathology similar to that seen in humans.[141,183,186] However, RSV protection studies in aged cotton, as a model for the elderly, are scarce. One previous study showed that nine-month-old cotton rats have a more prolonged RSV infection compared to young cotton rats.[215] This coincides with a delay in cytokine expression patterns in the lungs of infected cotton rats.[216] These data indicate that aged cotton rats indeed show signs of compromised antiviral immunity and would be a suitable model to study immune responses induced by RSV vaccines, including (MPLA-adjuvanted) RSV virosomes.

Here, we investigated the efficacy and safety of an RSV virosomal vaccine in old cotton rats that were either naïve to RSV or primed for RSV-specific immunity by infection early in life, similar to the general situation in humans. We show that intramuscular (IM) injection of RSV virosomes induce protective antibody responses in aged cotton rats and that incorporation of MPLA in the virosomes significantly boosts these responses without priming for enhanced

lung pathology. Immunity to RSV induced by infection early in life, as demonstrated by RSV-specific serum antibodies, waned significantly but protective antibody levels were still detected at old age. Levels of RSV-specific serum antibodies could, however, be boosted by IM injection of RSV virosomes, whether or not adjuvanted with MPLA. Finally, levels of respiratory tract RSV-specific secretory IgA (S-IgA), a correlate of protection against RSV in the elderly, could be boosted by intranasal (IN) administration of RSV virosomes to primed and aged cotton rats and incorporation of MPLA in the virosomes boosted this response. These data together suggest that RSV-MPLA virosomes have the capacity to boost protective RSV-specific immune responses in an aged immune system. Therefore, RSV-MPLA virosomes represent a promising vaccine candidate RSV vaccine for the elderly.

Results

RSV-MPLA virosomes in RSV-naïve old cotton rats

To determine whether RSV virosomes or RSV-MPLA virosomes are immunogenic in cotton rats with an aged immune system, aged (i.e. 8-months-old) cotton rats were immunized twice intramuscular (IM), three weeks apart, with RSV virosomes or RSV-MPLA virosomes. As controls, we included cotton rats that received no immunization or were immunized with FI-RSV by IM injection. We then analyzed levels of RSV-specific serum IgG, virus-neutralization capacities of sera obtained from the immunized cotton rats and protection against infection. Immunization with RSV virosomes in aged cotton rats gave rise to RSV-specific serum IgG with a geometric mean titer (GMT) of 2.7 (Figure 6.1A). Incorporation of MPLA in the virosomes significantly enhanced the levels of RSV-specific serum IgG. These levels were comparable to those induced by FI-RSV. A second immunization further stimulated IgG responses in the group vaccinated with RSV virosomes, but the levels were lower than those induced by two immunizations with RSV-MPLA virosomes or FI-RSV (Figure 6.1A).

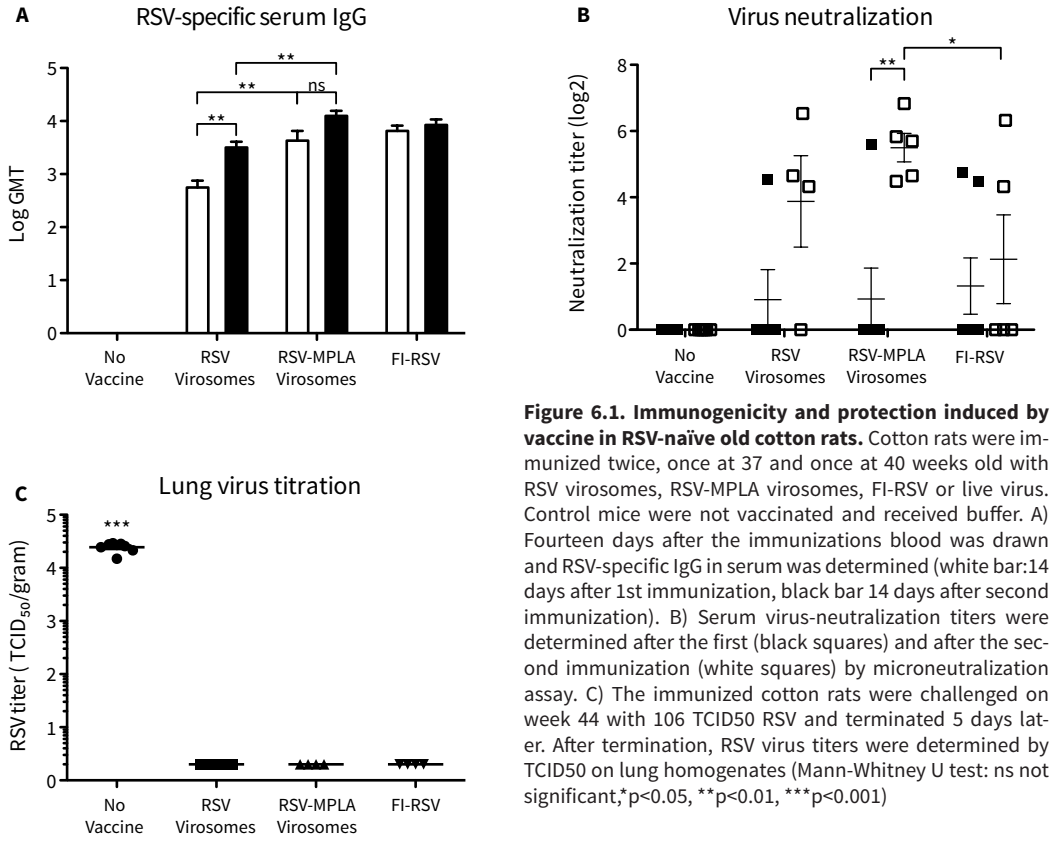


Figure 6.1. Immunogenicity and protection induced by vaccine in RSV-naïve old cotton rats. Cotton rats were immunized twice, once at 37 and once at 40 weeks old with RSV virosomes, RSV-MPLA virosomes, FI-RSV or live virus. Control mice were not vaccinated and received buffer. A) Fourteen days after the immunizations blood was drawn and RSV-specific IgG in serum was determined (white bar:14 days after 1st immunization, black bar 14 days after second immunization). B) Serum virus-neutralization titers were determined after the first (black squares) and after the second immunization (white squares) by microneutralization assay. C) The immunized cotton rats were challenged on week 44 with 106 TCID50 RSV and terminated 5 days later. After termination, RSV virus titers were determined by TCID50 on lung homogenates (Mann-Whitney U test: ns not significant,*p<0.05, **p<0.01, ***p<0.001)

Antiviral antibody titers in the sera were also analyzed in an *in vitro* micro-neutralization assay (Figure 6.1B). A single immunization with RSV virosomes, RSV-MPLA virosomes or FI-RSV induced detectable neutralizing antibody titers only in a fraction of animals (Fig 6.1B). After a second immunization, the number of animals with detectable neutralizing antibody titers increased in the groups that received RSV virosomes, but not in the group that received FI-RSV. However, all animals in the RSV-MPLA-immunized group then developed neutralizing antibodies (Figure 6.1B).

The aged and immunized cotton rats were challenged with live RSV, 49 days after the first immunization, to analyze the protection afforded by vaccination. After five days, the animals were sacrificed and virus titers were measured in the lungs (Figure 6.1C). The lungs from non-immunized animals all had virus titers of, on average, 4.39 log $TCID_{50}$. Cotton rats that received FI-RSV, RSV virosomes or RSV-MPLA virosomes did not have detectable lung virus titers (Figure 6.1C).

Enhanced respiratory disease

To determine the safety of RSV and RSV-MPLA virosomes with respect to immunopathology upon RSV infection following vaccination, we performed histopathological analyses on the lungs of immunized and challenged aged cotton rats. We compared the lungs from animals immunized with RSV virosomes and RSV-MPLA virosomes to the lungs of animals immunized with FI-RSV, which is known to prime for enhanced lung pathology.[140] The lungs from non-immunized aged cotton rats that were infected with RSV showed no signs of lung pathology: there were no alveolar infiltrates and no infiltration in the peribronchial and perivascular regions (Figure 6.2A). In contrast, aged cotton rats immunized with FI-RSV showed severe lung inflammation with peribronchial and perivascular infiltrates upon infection (Figure 6.2B). High numbers of neutrophils were also visible in the alveoli close to the bronchi. The lungs of the animals immunized with RSV virosomes showed some infiltrates in the peribronchial regions but no perivasculitis or alveolitis, as observed in animals immunized with FI-RSV. The lungs of aged cotton rats immunized with RSV-MPLA virosomes were similar to the lungs from non-immune animals and showed no signs of immunopathology upon infection (Figure 6.2D).

RSV virosomes in aged cotton rats infected with RSV early in life

The above data indicate that RSV-naïve aged cotton rats have an intact immune response, and are susceptible priming for enhanced disease like young cotton rats (Chapter 4). Practically all humans are exposed to RSV during childhood, and develop anti-RSV antibodies that remain present throughout life. To mimic this process, 27 day-old cotton rats were infected with RSV and, immunized with RSV virosomes 8 months later with or without MPLA as an adjuvant. Inoculation of live RSV to the young cotton rats induced an infection in all animals, as evidenced by the presence of RSV-specific serum IgG in all animals, 4 weeks after infection (Figure 6.3A). The levels of RSV-specific serum IgG were significantly decreased after 8 months, but still

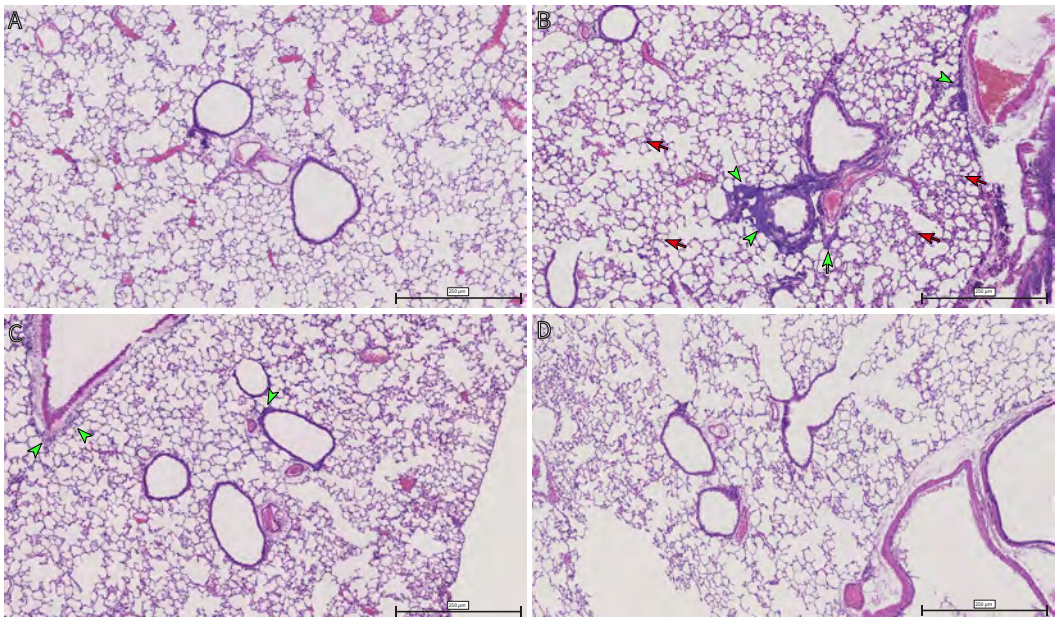


Figure 6.2. Lung pathology after immunization and challenge in old cotton rats. The cotton rats were immunized challenged and terminated as in figure 6.1 with A) buffer, B) FI-RSV, C) RSV virosomes and D) RSV-MPLA virosomes. The lungs were inflated and fixed with 4% formaldehyde and slices were stained with H&E. Lung pathology presenting as alveolitis and perivascular and peribronchial infiltrations was determined by light microscopy. Red arrows, alveolar infiltrates, green arrows, perivascular infiltrates, green arrowheads, peribronchial infiltrates.

detectable in all animals (Figure 6.3A). At that time, the animals received a single IM injection with RSV virosomes or RSV-MPLA virosomes. A control group was not immunized. Three weeks after immunization, we observed significant increases in RSV-specific serum IgG levels with respect to pre-immunization levels (Figure 6.3B). RSV-specific serum IgG was not different for animals immunized with virosomes with or without adjuvant (Figure 6.3B). We also observed a significant increase in virus-neutralizing capacity of sera from the animals that were immunized with RSV virosomes (Figure 6.3C). The virus-neutralizing capacity of sera from RSV-MPLA-immunized animals also increased, but the enhancement did not reach a statistically significant difference, probably due to a higher base-line neutralizing titer in pre-immunization sera from animals that received RSV-MPLA virosomes (Figure 6.3C).

Three weeks after immunization, the animals were challenged and five days later, RSV titers were determined in the lungs of the animals. RSV virus was not detected in the lungs from any group (Figure 6.3D). Therefore, RSV infection in young cotton rats induces immunity capable of protection against infection at 9-10 months.

Induction of S-IgA by intranasal immunization in old seropositive cotton rats

As mucosal S-IgA has also been linked with protection against RSV in the elderly,[214] we determined if local intranasal administration of RSV virosomes or RSV-MPLA virosomes could

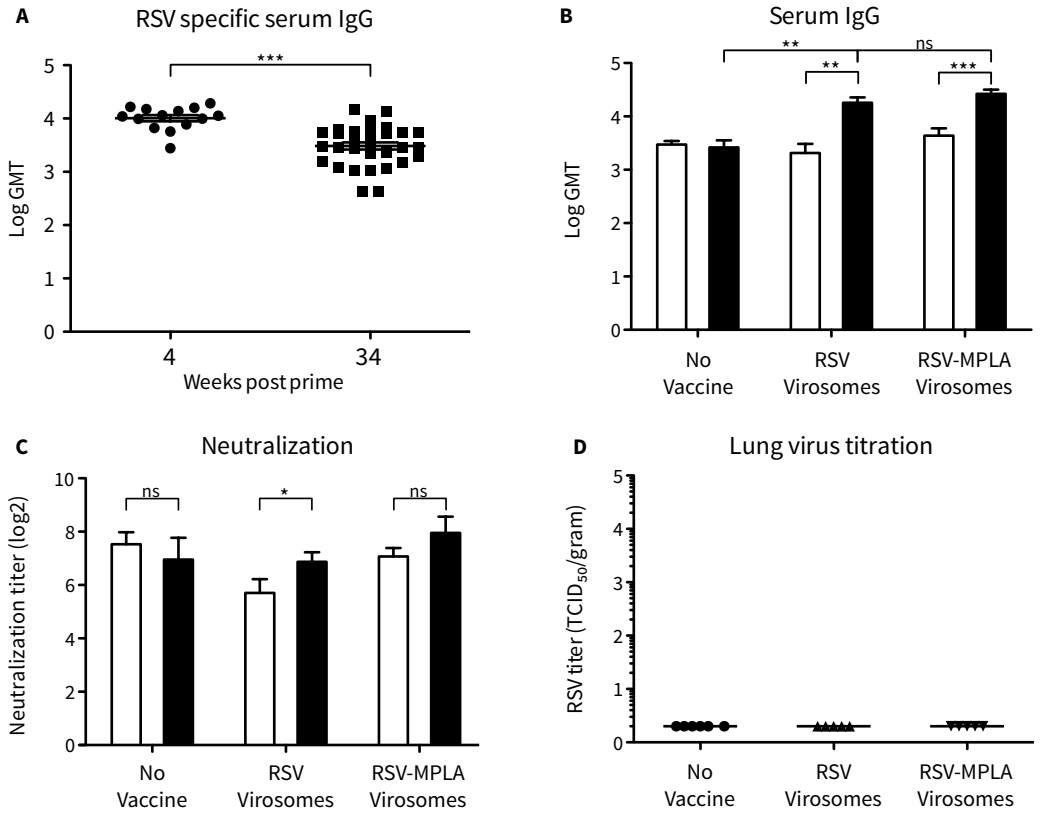


Figure 6.3. Immunogenicity and protection in previously infected, old cotton rats. Cotton rats were infected with RSV at the age of 27 days. A) Four and 34 weeks after infection blood was drawn to determine RSV-specific serum IgG in 14 and all the animals respectively. At 37 weeks, the cotton rats were immunized with RSV virosomes or RSV-MPLA virosomes. Control cotton rat received buffer. B, C) Three weeks after immunization blood was drawn for RSV-specific serum IgG and virus neutralization determinations before (white bars) and after (black bars) immunization. The cotton rats were challenged with 106 $TCID_{50}$ RSV. D) Five days after challenge the cotton rats were terminated and RSV virus titers were determined in lung homogenates. (Mann-Whitney U test: ns not significant,*p<0.05, **p<0.01, ***p<0.001)

boost mucosal S-IgA responses in primed and aged cotton rats. To this end, two groups of RSV-primed and aged cotton rats were immunized IN with RSV virosomes or RSV-MPLA virosomes and compared to groups that received the vaccine IM. Four weeks after immunization, lung and nasal washes as well as serum samples were taken to determine RSV-specific antibodies (Figure 6.4). IN immunization with RSV virosomes or RSV-MPLA virosomes resulted in significantly higher levels of S-IgA in lung and nasal washes when compared to those in washes from cotton rats that received the vaccine IM (Figure 6.4A and B). Incorporation of MPLA in IN-administered virosomes significantly boosted lung S-IgA levels, but not nasal S-IgA levels (Figure 6.4A and B). IN immunization with RSV virosomes, adjuvanted or not, did however not stimulate RSV-specific serum IgG responses (data not shown).

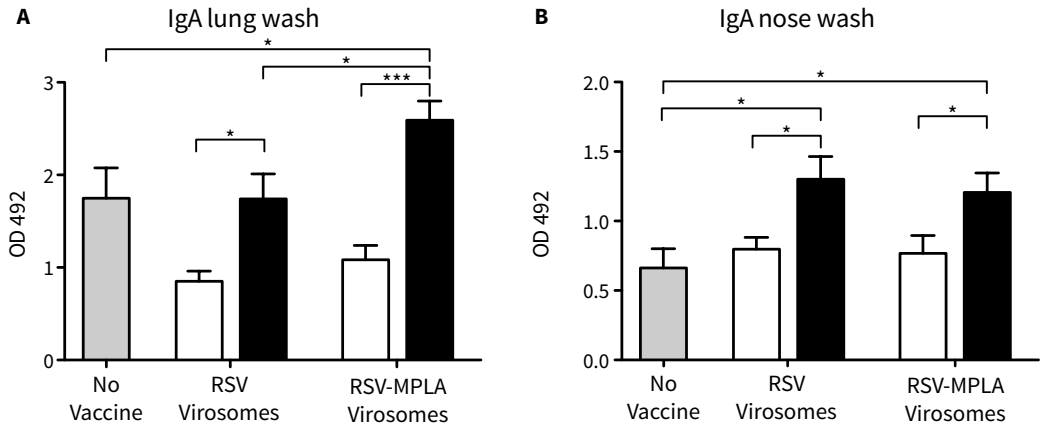


Figure 6.4. Mucosal IgA response in seropositive rats immunized with RSV virosomes and RSV-MPLA virosomes. The cotton rats were infected and aged as in figure 4. Subsequently the cotton rats were immunized with RSV virosomes and RSV-MPLA virosomes either intramuscularly (white bars) or intranasally (black bars). Control animals received buffer (grey bars). Three weeks after immunization the cotton rats were challenged and terminated. After termination, A) lung and B) nose washes were taken for RSV-specific IgA determination.

Discussion

An efficacious RSV vaccine could significantly decrease morbidity and mortality in the elderly. Immunosenescence in the elderly causes them to gradually become more susceptible to infections and, at the same time, less responsive to vaccinations.[217,218] Here we show that RSV virosomes and RSV virosomes adjuvanted with MPLA are efficacious and safe in boosting RSV-specific immunity in aged cotton rats.

The adjuvant effect of the TLR4 ligand MPLA on vaccine-induced immune responses in RSV-naive aged cotton rats is similar to the effect seen in young cotton rats (Chapter 4). This means that TLR4-mediated signaling is still intact in aged cotton rats. The available knowledge of TLR expression and function in the aged immune system is mainly derived from studies in mice which have shown decreased expression[219] or decreased function of several TLR, including TLR4.[220,221] One study in elderly people did show reduced a cytokine response to TLR1/2 ligands, but not to TLR4 ligands.[222] Therefore, differences may exist in the responses to different TLR ligands in aged immune systems from different species. An intact response to the TLR4 ligand MPLA in aged cotton rats, as shown in this study, but also in elderly people (17), suggests that MPLA is suitable as an adjuvant in RSV vaccines for the elderly.

Cotton rats that experienced an RSV infection early in life did not become susceptible for RSV at old age. We did observe a decline in serum IgG levels upon aging, however, this reduction was smaller than the decline reported in humans after infection[59] and the reduced levels if IgG were still able to afford protection. Co-evolution of RSV and the human immune system produced RSV strains capable of evading the human immune response to leave a previously infected person susceptible for subsequent infection. This is often referred to as 'incomplete' immunity induced by natural infection. Two possible mechanisms by which RSV inhibits the formation of effective immunity could involved inhibition of induction of type-1 IFN[11,12] or skewing the immune response to infection to a Th2-phenotype.[41] It is not clear if similar mechanisms are operational in RSV infection of cotton rats. Previous studies in cotton rats have shown, however, that RSV infection does not lead to a Th2-skewed response.[141] Also, cotton rats primed early in life were still protected against infection at old age (Figure 6.3), which argues against the presence of an efficient immune evading mechanism of RSV in cotton rats.

In primed and aged cotton rats, a similar increase in RSV-specific serum IgG was seen after IM immunization with RSV virosomes or RSV-MPLA virosomes. MPLA, although effective in aged cotton rats (Figure 6.1 and 6.4), does not seem to further boost serum IgG responses. An explanation for this may be the relatively high antibody levels in aged cotton rats induced by the infection early in life. Although these levels declined upon aging, they remained sufficiently high to provide protection. It is known that pre-existing antigen-specific antibodies negatively influence activation of B cells with the same antigen specificity, thereby avoiding unnecessary excess antibody production. This mechanism is also operational after influenza vaccination. For

example, vaccination with a similar strain as that used in the preceding years reduces further increases in antibody levels to that strain.[223] It is therefore likely that an enhancing effect of MPLA on serum antibody levels can only be observed when levels have sufficiently waned. RSV-MPLA virosomes administered IN to primed and aged cotton rats induce significant increases in respiratory tract S-IgA. A study in elderly people has shown that the levels of mucosal S-IgA correlated with protection against RSV infection.[214] Induction of S-IgA by vaccination is most efficiently induced by mucosal vaccination, e.g. IN immunization.[224] As the function of the nasal-associated lymphoid tissue remains relatively intact during aging, mucosal vaccination through the intranasal route seems attractive in, for example, approaches to induce (mucosal) immunity to RSV in the elderly.[225] In this respect, TLR ligands show promise as adjuvants in mucosal vaccines for the elderly.[225] In line with this, we found that the TLR4 ligand MPLA has the capacity to boost lung S-IgA responses upon IN immunization of aged cotton rats (Figure 6.4).

Taken together our data indicate that RSV virosomes adjuvanted with MPLA have the capacity to induce and boost RSV-specific protective immune responses in an aged immune system. These data warrant further exploration of MPLA-adjuvanted RSV virosomes as a candidate vaccine for risk groups such as the elderly.

Materials and Methods

Ethical statement

Animal experiments were approved by the Committee for Animal Experimentation (DEC) of the University Medical Center Groningen, according to the Dutch Animal Protection Act (permit number DEC 5266E). Immunizations and challenges were conducted under isoflurane anesthesia, and every effort was made to minimize suffering of the animals.

Cells and virus

RSV strain A2 (ATCC VR1540) was kindly donated by Mymetics BV (Leiden, The Netherlands). The virus was grown in Vero cells (ATCC, CL-81, Wesel Germany) :in SMF-4 Megavir (Thermo scientific, Etten-leur, The Netherlands), supplemented with L-glutamine, and penicilline/streptomycin (Invitrogen, Breda, Netherlands). After 5 days of infection the cell debris was cleared by low-speed centrifugation, the virus was concentrated and frozen in liquid nitrogen and stored at -80˚C to be used for live virus immunizations, challenge and microneutralization assays.

Vaccine formulations

RSV virosomes were generated as described previously.[168] Briefly, the RSV membrane was dissolved in 100 mM 1,2 dihexanoyl-*sn*-glycero-3-phosphocholine (DCPC) in HNE and the nucleocapsid was removed by ultracentrifugation. The supernatant was applied to a dried film of a 2:1 mixture of egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE) (Avanti Polar Lipids, Alabaster, AL, USA) at a ratio of 850 nmol lipid per mg of supernatant protein. For incorporation of MPLA, monophosphoryl lipid A from *Salmonella minnesota* Re 595 (Invivogen, Toulouse, France), was converted to 3-O-desacyl monophosphoryl lipid A by alkaline hydrolysis,[226] dissolved in 100 mM DCPC in HNE, and added to the protein/lipid mixture at 1 mg MPLA per mg supernatant protein, incubated for 15 min at 4˚C, filtered through a 0.1 µm filter and dialyzed in a sterile Slide-A-lyzer (10 kD cut-off; Thermo Scientific, Geel, Belgium) against HNE buffer pH 7.4. After dialysis, virosomes were kept at 4˚C.

FI-RSV was produced as reported before.[183] FI-RSV was diluted with HNE to contain 5 µg of RSV protein in a 50 µl volume.

Animals and immunizations

Female outbred cotton rats (Hsd:Cotton Rat) of 18 to 21 days old were obtained from Harlan (Indianapolis, IN, USA). One group of animals was housed conventionally for 34 weeks before immunization. Another group of animals received an infection with 10^6 $TCID_{50}$ live RSV and was housed in individually ventilated cages (IVC). The infected animals showed no signs of illness after infection. Blood was sampled at 4 weeks post infection to determine the RSV-specific IgG response. In a number of animals, aging was associated with development of disease symptoms including damaged eyes, abscess or tumor formation or group B Streptococcus infection of the uterus. Animals with more severe symptoms were terminated and excluded from the

experiment. RSV infection at a young age did not correlate with the onset of the symptoms.

Cotton rats that were not infected at a young age were immunized at the age of 37 weeks (priming immunization) and 40 weeks (boosting immunization) with RSV virosomes or RSV-MPLA virosomes intramuscularly (50 µl). Virosome preparations contained 5 µg of viral protein. Control rats received 50 µl of HNE intramuscularly or 50 µl (diluted to contain 5 µg viral protein) of FI-RSV intramuscularly. At week 44, cotton rats were challenged with 10^6 $TCID_{50}$ RSV intranasally. Cotton rats that received a primo infection were immunized once, at the age of 37 weeks with virosomes as described above. For the infected cotton rats, we also induced intranasal immunization with virosomes (100 µl). At 41 weeks, the cotton rats were challenged as described above.

At the time of immunization and challenge, blood was drawn by retro-orbital puncture. Five days after challenge, the cotton rats were sacrificed and blood was drawn by heart puncture. The cotton rats were resected and a small incision was made in the trachea. From this incision, nose washes and broncho-alveolar lavages were performed with 1 ml of PBS with protease inhibitors (Roche, Mannheim, Germany). Lungs were removed aseptically and one of the primary bronchi was ligated just below the tracheal bifurcation with suture wire. One half of each lung was kept on ice in HEp2 medium containing 2% FBS, for virus titration. The other half of each lung was fixed in 4% formaldehyde in PBS at 20 cm of water pressure to preserve the structure of the lungs for lung histopathological analyses.

IgG antibody ELISA

RSV-specific antibody titers were determined as described before.[168] Briefly, 96-well plates were coated with betapropiolactone-inactivated RSV and blocked with 2.5% milk powder in coating buffer. Plates were then incubated for 90 min with two-fold serial dilutions of serum, BAL or nose washes, starting at dilutions of 1:200 for serum or 1:1 for BAL or nose washes. After washing, plates were incubated with a 1:5000 dilution of horseradish-peroxidase-coupled goat anti-mouse IgG, or IgA which are cross reactive with cotton rat IgG and IgA, respectively (Southern Biotech 1030-05, 1040-05) for 1 h and subsequently stained with *o*-Phenylenediamine (OPD; Sigma-Aldrich, St Louis, MO, USA). After 30 min the staining was stopped by addition of 2 M H_2SO_4 and absorption was measured at 492 nm.

Virus titration and microneutralization assay

Virus titers were determined by $TCID_{50}$ as described previously.[185] For determination of RSV virus neutralization titers, serum was decomplemented by heat inactivation for 30 min at 56˚C. Neutralization titers were determined by incubation of two-fold serially diluted decomplemented serum with 70 $TCID_{50}$ of RSV for 2 h and subsequent titration of this mixture on HEp-2 cells as described before.[185] The neutralization titer was calculated with the Reed & Muench method as the dilution that neutralizes infection in 50% of the wells.

Lung histopathology

The inflated lungs were embedded in paraffin and 4 µm slices were cut. The slides were then stained with hematoxylin and eosin (H&E) using standard procedures. Subsequently, perivasculitis, peribronchiolitis and alveolitis were assessed by light-microscopy.

Statistical analyses

All statistical analyses were performed with Graphad Prism 5.00 for Mac OSX, (GraphPad Software, San Diego California USA; www.graphpad.com). Statistical significance was assessed using a Mann-Whitney U test. P values of 0.05 or lower were considered to represent significant differences.

Chapter 7

Summarizing discussion and future perspectives

Tobias Kamphuis[1], Jan Wilschut[1], Aalzen de Haan[1]

1) Department of Medical Microbiology, Molecular Virology Section, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

Even though RSV infection remains a serious threat to newborns and the elderly, no vaccine against RSV is available today. Here, we set out to develop a safe and efficacious, inactivated RSV vaccine based on "virosomes", reconstituted RSV envelopes containing the viral membrane glycoproteins but lacking the nucleocapsid, with built-in lipophilic Toll-like receptor (TLR) ligands as adjuvants. First, we developed a procedure for production of RSV virosomes from purified virus. During reconstitution we incorporated the TLR2 ligand Pam3CSK4 or the TLR4 ligand MPLA as immunomodulating adjuvants. Immunogenicity and safety of these these preparations were evaluated in mice and cotton rats. Additionally, the ability of RSV-MPLA virosomes to induce a protective immune response after intranasal administration was examined and finally, the immunogenicity of RSV-MPLA virosomes in an aged immune system was determined.

RSV virosomes

In **Chapter 2** we showed that it is feasible to generate virosomes directly from purified RSV. In mice, RSV virosomes induced higher levels of IgG1 compared to IgG2 and did not stimulate production of IFN-γ in splenocytes, suggesting that this preparation skews immune responses to a Th2-phenotype. The addition of the TLR2 ligand Pam3CSK4 however, increased both IgG2a and IFN-γ levels, demonstrating the ability of virosome-incorporated TLR ligands to skew the immune response to a more favorable, balanced Th1/Th2-phenotype. In a previous study, adjuvanted RSV virosomes were produced by inserting purified RSV F protein into influenza virosomes mixed with *E. coli* heat-labile enterotoxin (HLT), as an adjuvant. These virosomes however, induced a Th2-skewed immune response in mice, as was indicated by the induction of approximately 20-fold higher levels of IgG1 compared to IgG2.[148] Lipophilic TLR ligands, such as for example Pam3CSK4, may be better suited for use in RSV virosomal vaccines, not only because they skew responses to a favorable Th1-phenotype, but also because they allow tight association with the virosomal membranes. This association of antigen and adjuvant results in activation of only those antigen-presenting cells, that take up and process the antigen. This reduces undesirable activation of bystander cells by adjuvant only. The addition of TLR ligands to an RSV vaccine does not only lead to a more balanced Th1/Th2 response but additionally leads to the induction of antibodies with higher neutralizing capacity, possibly through improved antibody affinity maturation. In the early FI-RSV clinical trials, the induced antibodies failed to neutralize the virus upon infection;[72] this later on appeared to have been due to deficient TLR signaling by FI-RSV.[76] Inclusion of Pam3CSK4 into the RSV virosomal vaccine therefore improves both its safety and its efficacy to induce neutralizing antibodies.

RSV-MPLA virosomes

Monophosphoryl lipid A (MPLA) is a TLR4 ligand that is already in use in vaccines licensed for use in humans and has an acceptable safety record. MPLA, like Pam3CSK4, is lipophilic which would allow incorporation in virosomal membranes. However, MPLA possesses superior Th1-skewing properties compared to Pam3CSK4. This prompted us to explore the use of MPLA as an adjuvant in RSV virosomes. In **Chapter 3**, we determined the immunoactivating properties of RSV-MPLA

virosomes *in vitro* and *in vivo*. Using TLR4-expressing cell lines with NFκB reporter genes, we showed that RSV virosomes have TLR4-activating capacity, and that this capacity is enhanced by the incorporation of MPLA into the virosomal membrane. Although RSV-F in RSV virosomes activates TLR4 (Chapter 3), and has been shown to induce inflammatory cytokines such as for instance IL-6,[42] immunization with RSV virosomes without MPLA does not lead to a Th1-skewed immune response (Chapter 3). This could be due to the magnitude of TLR4 activation by RSV virosomes, which is clearly lower compared to that of RSV-MPLA virosomes. Alternatively, MPLA may signal through a different TLR4-adaptor molecule than the one used by RSV-F.[205] MPLA mediated TLR4-signaling occurs through adaptor molecule TRIF,[169] while signaling mediated by RSV-F most likely occurs through MyD88. Signaling through TRIF increases the induction of type-I IFN.[169] High levels of type-I IFN induced by MPLA lead to Th1-polarization.[38]and also contribute to IgG class switching and antibody affinity maturation,[177,180] which both contribute to stronger and safer immunity to RSV. Possibly, MPLA in RSV virosomes competes with RSV-F for binding to TLR4. This could result in diminished RSV-F-induced MyD88 mediated TLR signaling and to lower production of inflammatory cytokines in favor of higher type-I IFN levels induced by MPLA.

In addition to experiments in mice, we examined the safety and protective capacity of RSV-MPLA immunizations in cotton rats (**Chapter 4**). Cotton rats were used because they are more permissive for RSV infections than mice and closely replicate human enhanced disease parameters that were found in the FI-RSV clinical trial.[140,144] Local Th2-cytokine expression levels and lung pathology consisting mainly of neutrophilic infiltrates were measured as indicators of ERD. In contrast to immunization with FI-RSV, neither immunization with RSV virosomes nor RSV-MPLA virosomes gave rise to an increase in Th2-cytokine expression in the lungs of the animals after challenge. There were however, significantly more neutrophilic infiltrates in the lungs of cotton rats that received RSV virosomes compared to those that received RSV-MPLA virosomes. These infiltrates may have been attracted as a consequence of the induction of poorly neutralizing antibodies by RSV virosome immunizations. Poorly neutralizing antibodies can form immune complexes in the lungs which can bind to and activate complement factors attracting neutrophils.[227] Complement factor C3a has also been found in the lungs of the children who died in the 1960s FI-RSV trial indicating that complement activation by immune complexes could have contributed to the induction of ERD.[74] The incorporation of MPLA in RSV virosomes does not only induce a balanced Th1/Th2 response but, importantly, also is likely to enhance antibody affinity maturation,[76] leading to the induction of highly neutralizing antibodies without formation of immune complexes and induction of ERD.

Alternative routes of vaccine administration

Intranasal (IN) administration is an acceptable route of vaccination and could, in addition to systemic immunity, also induce local immune responses, including production of secretory IgA (S-IgA). As the main port of entry of RSV is the mucosa of the respiratory tract, an RSV vaccine

would benefit from induction of a mucosal immune response. In **Chapter 5**, we therefore set out to determine the immunogenicity and protective capacity of intranasally administered RSV-MPLA virosomes. In mice, intranasally administered RSV virosomes induced high systemic and mucosal antibody responses, similar to immunizations with live virus. In cotton rats, however, IN vaccination appeared less potent in inducing mucosal antibody responses, particularly when compared to responses induced by immunization with live virus. However, cotton rats are more permissive for RSV than mice.[141] Therefore, the local immune activation and the resulting antibody response induced by infection could be much stronger in cotton rats than in mice which could explain the relative differences between the immune response induced by RSV-MPLA virosomes and live virus infection seen in mice versus cotton rats. Interestingly, some LPS-derived TLR4 ligands have been shown to be species-specific in their capacity to activate TLR4.[228] Such a species-specific activation probably does not play a role in the different efficacy of MPLA to enhance mucosal antibody responses in mice versus responses in cotton rats: MPLA effectively stimulated serum antibody responses in both species (Chapter 5) and is effective in humans too.

In mice, MPLA demonstrated a clear mucosal adjuvant effect, as seen by increased S-IgA responses. Interestingly, expression of the polymeric IgG receptor (pIgR), a receptor responsible for transcytosis of polymeric S-IgA, is enhanced by TLR ligands through activation of MyD88.[210,211] Although MPLA could increase expression of pIgR and thereby boost transcytosis of polymeric IgA, this may be only a transient effect that would wane rapidly after vaccination. Therefore, the mucosal adjuvant activity of MPLA may rather be due to local activation of antigen presenting cells and B cells leading to an enhanced local SIgA antibody response. In adult humans, mucosal IgA levels also have been shown to correlate with protection against infection.[214] Therefore, induction of these responses would further improve vaccine-mediated RSV-specific immunity.

RSV vaccination in the elderly

The elderly are at significant risk of severe RSV infections.[143] It is known that aging reduces immunocompetence, and therefore it is of interest to study the immunogenicity of RSV virosomes, as well as the immunoadjuvant activity of MPLA in an aged immune system. In **Chapter 6** we showed that RSV-MPLA virosomes have the capacity to induce protective RSV-specific immunity in aged cotton rats. The adjuvant effect of MPLA was similar to that seen in young cotton rats, indicating that TLR4 signaling in aged cotton rats is still effective. In mice, the TLR4 signaling capacity decreases with age,[220,221] however, in humans it remains active upon aging[222] like in aged cotton rats.

In humans, RSV infection early in life does not lead to lifelong protection. There is however detectable humoral immunity against RSV in elderly people.[229] To assess if RSV-MPLA virosomes can boost antibody responses in a situation of pre-existing RSV-specific immunity, we infected cotton rats early in life and immunized them with virosomes at old age. However, live RSV-

primed cotton rats did not become susceptible to RSV infection upon aging. This means that infection of cotton rats with RSV induces an effective and durable immunity and that in this respect, the situation is different from that in humans. In humans, RSV employs immune evasive mechanisms such as inhibition of type-I IFN induction by NS proteins[11,12] and direction of the immune response to a Th2-phenotype.[41] These mechanisms may have developed during the long co-evolution of RSV and its natural host, i.e. humans, and may not be (fully) operational in cotton rats. Further research is necessary to evaluate if RSV-primed and aged cotton rats represent a good model to study the potential of a virosomal RSV vaccine, or other vaccine candidates, to boost immunity in an aged immune system. Perhaps, to more closely mimic the situation in humans, priming of RSV immunity in young cotton rats could be done by RSV infection that is confined to nasal cavity only, as opposed to a total respiratory tract infection used in our previous experiments.

Concluding remarks

Taken together, the data presented in this thesis demonstrate that RSV virosomes with built-in MPLA as an immunomodulating adjuvant, represent a promising candidate RSV vaccine. We have shown that RSV-MLA virosomes are immunogenic in mice and cotton rats inducing a balanced Th1/Th2 response and high levels of RSV-neutralizing antibodies. Safety aspects of a candidate RSV vaccine are equally important to immunogenicity. Immunization with RSV-MPLA virosomes does not lead to increases in Th2-type cytokines or increased pulmonary infiltrates in cotton rats upon subsequent exposure of the animals to live virus. Furthermore, it is likely that RSV-MPLA virosomes will be able to boost immune responses in previously RSV infected elderly people. Whether or not the robust immunogenicity will indeed translate into a reduction of RSV-related morbidity and mortality in humans will have to be assessed in clinical trials.

Two very different risk-groups can greatly benefit from an RSV vaccine: infants and the elderly. The immune responses in these two populations differ significantly and induction of adequate responses will probably require different vaccine approaches.[163] Because TLR signaling in infants takes up to six months to develop,[230,231] RSV-MPLA virosomes could be less suitable for immunization of this population. It is generally assumed that infants will benefit most from live attenuated, intranasally administered vaccines as these prime the immune system similarly to infection with live virus and do not give rise to ERD.[232] Immune responses induced by a good live attenuated vaccine could be robust and durable. These young infants however are also most susceptible to infectious RSV; therefore a live attenuated vaccine that reverts to wild type could induce significant disease in this risk-group.[84] A perhaps more safe alternative to the vaccination of infants would be maternal vaccination. In this approach, pregnant women are immunized and the induced RSV-specific antibodies transfer through the placenta to the unborn child.[233] Also, antibodies in breast milk are transferred to the newborns after birth.[233] Non-replicating (subunit) RSV vaccines, including RSV-MPLA virosomes, would be highly suitable for this approach.

For the elderly, an adjuvanted virosomal vaccine could be of great benefit. Incorporated TLR ligands as adjuvants have the capacity to increase the immunogenicity of the vaccine in the setting of an aged and less responsive immune system. If TLR ligands are needed to prevent priming for ERD in the elderly remains to be seen. Most likely, the elderly have been primed by RSV infection earlier in life and studies in mice have shown that earlier priming with live virus inhibits priming for ERD by vaccination.[234] Therefore, ERD may not be readily induced by immunization of the elderly with non-replicating RSV vaccines. Previous clinical trials evaluating subunit vaccines in adults have shown that these vaccines increase levels of neutralizing antibodies and reduce the overall incidence of all RSV infections.[235] However, no significant decrease in lower respiratory tract infection was observed.[235] This could point to the inability of these vaccines to induce cellular immune responses, important in protection of the lungs against RSV infection. In this respect, MPLA has been shown to enhance cellular responses in other vaccines[174,236] and this ligand probably also could improve cellular responses against RSV. Furthermore, TLR signaling by MPLA could not only improve overall immunogenicity of RSV virosomes but also lead to the induction of a longer lasting immune response compared to that induced by RSV vaccines without TLR adjuvant.[197] Whether or not RSV-MPLA virosomes indeed have the capacity to induce a more durable immune response in humans and also induce cellular immune responses will need to be investigated further.

Annex

Nederlandse samenvatting
Acknowledgements
Publication list
References

Nederlandse samenvatting

Infectie met het respiratoir syncytieel virus (RSV) is de voornaamste oorzaak van virale bronchiolitis (longsteking) bij jonge kinderen. Daarnaast is RSV-infectie ook verantwoordelijk voor een groot deel van de lagere-luchtweginfecties bij ouderen en mensen met een verzwakt immuunsysteem. RSV wordt al sinds de ontdekking van het virus in de jaren '50 van de vorige eeuw gezien als een belangrijk doel voor vaccinatie, maar tot op heden is er nog geen RSV-vaccin op de markt. De oorzaken hiervan lopen uiteen van het ontbreken van een goed diermodel om experimentele vaccins in te testen, en de onvolledige bescherming die een infectie met levend RSV met zich meebrengt, tot de dramatische uitkomst van een klinische studie met een formaline-geïnactiveerd RSV-vaccin in de jaren '60 van de vorige eeuw.

RSV komt het lichaam binnen via de luchtwegen. Dat kan gebeuren door contact met een geïnfecteerd persoon of door contact met gecontamineerde oppervlakken van bijvoorbeeld speelgoed. Eenmaal in de luchtpijp en de bronchiën infecteert RSV het epitheel en kan daar vervolgens repliceren. RSV verspreidt zich binnen een individu door het vrijkomen van infectieus virus uit geïnfecteerde epitheelcellen, dat weer nieuwe cellen infecteert, maar ook door het fuseren van geïnfecteerde cellen met nog niet geïnfecteerde cellen. Dit fusieproces wordt teweeg gebracht door het virale fusie-eiwit dat op de membraan van geïnfecteerde cellen tot expressie wordt gebracht en leidt tot het ontstaan van grote klompen gefuseerde cellen, ook wel syncytia genoemd. De virale infectie en het ontstaan van syncytia worden gedetecteerd door het immuunsysteem dat vervolgens het virus uitschakelt en de geïnfecteerde cellen opruimt. In de meeste gevallen duurt de ziekte niet lang en verdwijnen de symptomen na ongeveer een week. In ergere gevallen kan het virus zich verspreiden naar de lagere luchtwegen en hier een longontsteking veroorzaken. Bij mensen met een verzwakt immuunsysteem kan dit de dood tot gevolg hebben.

Na het succes van het geïnactiveerde poliovaccin in de jaren '50 en later, is er ook een poging gedaan om een formaline-geïnactiveerd RSV-vaccin (FI-RSV) te maken. Halverwege de jaren '60 is dit vaccin getest in een drietal klinische studies bij jonge kinderen. Het FI-RSV leek aanvankelijk goed te werken: het induceerde een afweerreactie met vorming van RSV-specifieke antistoffen. Deze antistoffen zouden bescherming moeten bieden tegen infectie met RSV. Echter, toen een aantal van de gevaccineerde kinderen een RSV-infectie doormaakten, bleken zij vatbaarder te zijn geworden voor het virus dan de niet-gevaccineerde kinderen in de controlegroep. Vooral de jongste kinderen, die voor de vaccinatie nog seronegatief waren voor RSV, maakten een verergerde vorm van RSV-infectie door. Dit leidde tot een toename in ziekenhuisopnames, terwijl twee van de jongste kinderen in de gevaccineerde groep zelfs kwamen te overleiden.

De immunologie stond in de jaren '60 en '70 nog in de kinderschoenen en het heeft dan ook lang geduurd voordat onderzoekers precies wisten waardoor FI-RSV niet in staat was de kinderen te beschermen tegen RSV, maar de ziekte juist verergerde. Weliswaar werd snel duidelijk dat

de antistoffen die aanwezig waren bij de gevaccineerde kinderen niet in staat waren het virus te neutraliseren. Maar pas veel later is aan het licht gekomen dat dit veroorzaakt werd door een inadequate vorm van zogenaamde “affiniteitsmaturatie”; dit is het proces dat er normaliter voor zorgt dat antistoffen een hoge affiniteit ontwikkelen voor de antigenen waartegen ze zijn gericht. Dit bleek te komen doordat FI-RSV in onvoldoende mate “Toll-Like” receptoren (TLR) stimuleert, die betrokken zijn bij het sturen van de immunologische afweereactie en ook bijdragen aan affiniteitsmaturatie van antistoffen. Tevens is uit dierstudies gebleken dat FI-RSV een afweerreactie met een zogenaamd Th2-fenotype induceert.

De immunologische afweereactie tegen infectieuze ziekteverwekkers kan ruwweg opgedeeld worden in een respons met een Th1- of een Th2-fenotype. Th2-type afweerreacties bestaan uit de productie van antistoffen van het subtype IgE en de activatie van onder andere neutrofielen en eosinofielen. Deze afweerreactie is bij uitstek geschikt voor de bescherming tegen extracellulaire bedreigingen zoals parasitaire en sommige bacteriële infecties. Een Th1-respons daarentegen wordt gekenmerkt door de vorming van antistoffen die virussen kunnen neutraliseren en door de activatie van cytotoxische T-cellen. Een dergelijke respons is dus meer geschikt voor bescherming tegen virussen zoals RSV.

Sinds de jaren ‘60 zijn er meerdere RSV-vaccinkandidaten getest in diermodellen en bij mensen, maar tot nu toe is er nog geen vaccin gevonden dat goed beschermt en – minstens even belangrijk - volkomen veilig is. Een mogelijke manier om op een veilige manier bescherming te bieden tegen RSV-infecties is het gebruik van RSV-virosomen. Een virosoom is als het ware een virusdeeltje zonder het genetische materiaal. Hierdoor ziet een virosoom er voor het immuunsysteem uit als het virus, maar is het niet in staat om te repliceren; een virosoom kan dus geen ziekte veroorzaken. Ons lab heeft eerder laten zien dat het mogelijk is om virosomen van influenza virus te maken door dit virus te behandelen met een soort zeep, een fosfolipide dat er voor zorgt dat het membraan oplost waardoor de membraaneiwitten in oplossing komen. Vervolgens wordt op basis van dichtheid het nucleocapside, waar het RNA van het virus in zit, verwijderd door ultracentrifugatie. Als het fosfolipide dan vervolgens weer wordt weggehaald door middel van dialyse vormt het virale membraan zich weer met daarin de virale membraaneiwitten. Verder is het mogelijk om, tijdens deze zogenaamde reconstitutie, lipofiele moleculen toe te voegen die dan ook in de virosomale membraan komen te zitten. Dit kunnen bijvoorbeeld TLR-liganden zijn die de afweerreactie tegen een vaccin kunnen stimuleren en een bepaalde kant op kunnen sturen. Momenteel zijn er al virosomen op de markt die gebruikt worden als vaccin tegen influenza. In deze studie hebben wij onderzocht of het mogelijk is om deze techniek ook toe te passen voor RSV.

Om tijdens een infectie een goede afweerreactie tot stand te laten komen moet het afweersysteem op meerdere manieren gestimuleerd worden. Het afweersytyeem herkent eiwitten van de ziekteverwekker en zal, als deze herkenning vergezeld gaat met een soort van activatiesignaal

een afweer reactie tegen deze eiwitten tot stand brengen. Deze activatiesignalen komen meestal van bepaalde lichaamsvreemde moleculen. Zo kunnen de aanwezigheid van bouwstenen van bacteriële celwanden of de aanwezigheid van dubbelstrengs RNA, iets dat in de mens in principe niet voorkomt, de afweerreactie tegen een infectie versterken. Deze acitvatiesignalen worden herkent door speciale receptoren op cellen van het immuunsyteem. In tegenstelling tot een infectie zal een vaccin dat bestaat uit bijvoorbeeld alleen een gezuiverd viraal eiwit deze activatiesignalen ontberen. Doordat echter bekent is welke signalen verantwoordelijk zijn voor het activeren van een bepaalde afweerreactie kunnen we dit soort signalen toevoegen aan een vaccin. Een stof die toe wordt gevoegd aan een vaccin wordt ook wel een adjuvant genoemd. Het inbouwen van een TLR ligand in onze RSV virosomen is een voorbeeld van het gebruik van een dergelijk adjuvant dat de afweerreactie kan versterken en bijsturen.

In hoofdstuk 2 laten we zien dat het inderdaad mogelijk is om virosomen te maken van gezuiverd RSV. Deze virosomen hebben we vervolgens gekarakteriseerd door middel van elektronenmicroscopie en eiwit-gelelectroforese. Met deze technieken hebben we ook laten zien dat het TLR2-ligand Pam3CSK4 is geassocieerd met de virosomen. Vervolgens hebben we muizen geïmmuniseerd met de virosomen en de humorale (antistoffen) en cellulaire (T-lymfocyten) immuunrespons gemeten. Hieruit bleek dat de RSV/Pam3CSK4-virosomen inderdaad een gebalanceerde Th1/Th2-immuun respons opwekken. De geïmmuniseerde muizen werden vervolgens geïnfecteerd met levend RSV en na vijf dagen bleek dat de geïmmuniseerde muizen in staat waren het virus uit te schakelen in tegenstelling tot de niet-geïmmuniseerde muizen. Om de veiligheid van het vaccin te controleren is er vervolgens een vaccinatie- en infectie-experiment gedaan in katoenratten. De katoenrat is vatbaarder voor RSV dan de muis en de reactie van katoenratten op het FI-RSV, dat in de jaren ‘60 tot verergerde ziekte leidde bij mensen, lijkt op de reactie die ook bij mensen wordt waargenomen. In dit experiment bleek dat immunisatie van katoenrattenmet FI-RSV, zoals verwacht, leidde tot verergerde ziekte in de longen na infectie. De katoenratten die geïmmuniseerd waren met RSV/Pam3CSK4-virosomen lieten daarentegen geen tekenen zien van verergerde longpathologie. Het lijkt er dus op dat RSV/Pam3CSK4-virosomen een geschikte RSV-vaccinkandidaat vormen.

Naast de TLR2-ligand Pam3CSK4 zijn er ook andere TLR-liganden die gekozen kunnen worden om in RSV-virosomen te verwerken. Het TLR4-ligand MPLA wordt al gebruikt in een aantal vaccins die op de markt zijn. MPLA heeft als potentieel voordeel ten opzichte van Pam3CSK4 dat het, doordat het via een andere route werkt dan Pam3CSK4, effectiever is in het opwekken van een Th1-type respons. Aangezien MPLA ook een lipofiel molecuul is hebben we geprobeerd om MPLA in virosomen in te bouwen.

In hoofdstuk 3 laten we zien dat het mogelijk is om MPLA in virosomen in te bouwen en dat deze virosomen ook daadwerkelijk activatie van TLR4 in cellen bewerkstelligen. Verder laten we zien dat immunisatie van muizen met RSV/MPLA-virosomen leidt tot een gebalanceerde Th1/

Th2-immuunrespons en dat de antistoffen die geïnduceerd worden door de immunisatie goed in staat zijn RSV te neutraliseren. Om het effect van MPLA in virosomen op het voorkomen van verergerde ziekte te beoordelen, hebben we in hoofdstuk 4 dit vaccin ook getest in katoenratten. RSV/MPLA-virosomen bleken in staat om de katoenratten te beschermen tegen RSV-infectie en, in tegenstelling tot immunisatie met FI-RSV, ging dit niet gepaard met de expressie van Th2-geassocieerde cytokines. RSV-virosomen zonder MPLA induceerden ook geen verhoogde expressieniveaus van deze cytokines, maar immunisatie van katoenratten met virosomen zonder adjuvant leidde toch tot symptomen van verergerde ziekte zoals de influx van neutrofielen in de long. Het adjuvant versterkt niet alleen de versterkt niet alleen de afweerreactie, maar stuurt deze ook in een veiligere richting en is daarom onontbeerlijk voor het goed functioneren van een virosomaal RSV-vaccin.

Een groot probleem van vaccinatie is dat er voor de toediening van de meeste vaccins naalden nodig zijn. Dit verhoogt de drempel voor mensen om een vaccin te nemen. Bovendien is er geschoold personeel nodig om een vaccin via een injectie toe te dienen. Een RSV-vaccin dat op een andere manier toegediend zou kunnen worden, bijvoorbeel via een nesusspray, zou daarom zeer welkom zijn. Daarnaast zou een RSV-vaccin erbij gebaat zijn als het lokale bescherming in de neus en luchtwegen zou bieden aangezien RSV via de luchtwegen zijn gastheer binnenkomt. Om te testen of RSV/MPLA-virosomen ook goed beschermen als ze intranasaal worden gegeven, hebben we in hoofdstuk 5 een aantal experimenten gedaan in muizen en katoenratten waar we dit aspect hebben geëvalueerd. Uit deze experimenten bleek dat RSV-virosomen zonder adjuvant nagenoeg geen immuunrespons opwekken als ze intranasaal worden toegediend. Dit verandert echter door het inbouwen van een adjuvant, al is de bescherming die wordt verkregen met intranasaal toegediende virosomen minder robuust dan na een intramusculaire injectie van RSV/MPLA-virosomen.

Er is een wezenlijk verschil tussen het vaccineren van dieren die nog nooit een RSV-infectie hebben doorgemaakt en het vaccineren van oudere mensen die gedurende hun leven al meerdere keren in aanraking zijn gekomen met RSV. Om dichter bij deze situatie in de buurt te komen hebben we in hoofdstuk 6 geprobeerd een model op te zetten waarbij katoenratten op jonge leeftijd worden geïnfecteerd met RSV en vervolgens ouder worden. Na negen maanden hebben we onderzocht of de katoenratten, net als oudere mensen, weer vatbaar worden voor het virus, of de bestaande afweerreactie gestimuleerd kan worden met RSV-virosomen en of hiervoor een adjuvant nodig is. In tegenstelling tot oudere mensen waar de immuniteit tegen RSV op een gegeven moment niet meer in staat is te beschermen tegen RSV-infectie, bleken de katoenratten negen maanden na de primaire infectie nog steeds beschermd te zijn. Ondanks dat in katoenratten de aanwezige immuunrespons niet genoeg bleek af te nemen om ze weer vatbaar te laten worden voor het virus, konden we wel zien dat de antistofniveaus afnamen in de tijd en dat deze door immunisatie met RSV- of RSV/MPLA-virosomen weer gestimuleerd konden worden. Verder bleek dat in oudere katoenratten de lokale afweer gestimuleerd kon worden

door intranasale toediening van het vaccin. Hierbij werd ook duidelijk dat RSV/MPLA-virosomen een grotere toename van lokale antistoffen induceren dan RSV-virosomen zonder adjuvant. Verder is uit de literatuur bekend dat het immuunsysteem van oudere mensen nog goed in staat is om adequaat op TLR4-liganden te reageren. Daarom zouden RSV/MPLA-virosomen een goed vaccin kunnen zijn om ziekte en sterfte ten gevolge van RSV-infectie bij ouderen te voorkomen. Of RSV/MPLA-virosomen bij mensen voor een voldoende toename in immuniteit kunnen zorgen en of deze immuniteit dan ook in staat is ouderen te beschermen tegen RSV zal moeten blijken uit klinische studies.

In dit onderzoek hebben we laten zien dat het mogelijk is omRSV-virosomen te maken en dat de afweerreactie die deze virosomen teweeg brengen sterk verbeterd kan worden door het inbouwen van de TLR-liganden Pam3CSK4 of MPLA in de virosomale membraan. De virosomen met TLR ligand zijn immunogeen, bieden bescherming tegen infectie in diermodellen en veroorzaken geen tekenen van verergerde ziekte. De positive resultaten uit deze studie geven aanleiding om het vaccin te testen in een klinische studie. Eerst zal hierbij worden gekeken naar of het vaccin goed getolereerd wordt en geen bijwerkingen veroorzaakt na injectie. Als dit allemaal goed veloopt zal worden onderzocht of de RSV virosomen, net als in muizen en in katoenratten een goede afweerreactie teweeg brengen en als laatst zal een grote studie aan moeten tonen of deze afweerreactie ook daadwerkelijk in staat is tegen RSV infectie te beschermen. Voordat het vaccin op de markt komt is er dus nog een lange weg te gaan, maar het is duidelijk dat vaccinatie met RSV virosomen met een TLR ligand als adjuvant een veelbelovende aanpak van dit belangrijke probleem is.

Acknowledgements

Ondanks het feit dat mijn naam de enige is die op de kaft van dit boekje staat was dit proefschrift nooit tot stand gekomen zonder de hulp en steun van een heleboel mensen.

Als eerst wil ik **Jan** bedanken voor de kans die hij mij heeft gegeven om in zijn lab mijn promotieonderzoek uit te voeren. Na mijn stage bij Anke en Judith heb je me tot twee keer toe gevraagd of ik bij de Moleculaire Virologie wilde promoveren. Ik was vereerd door het aanbod en toen het voor mij duidelijk werd, dat ik het motiverend vind dat mijn onderzoek een min of meer directe toepassing kan krijgen die bijdraagt aan het welzijn van mensen, was de keus om aan een RSV vaccin te gaan werken snel gemaakt. Ik heb onze gesprekken altijd als stimulerend ervaren en vooral op het eind van het traject, toen deze gesprekken wat regelmatiger plaatsvonden, heeft het me erg veel geholpen. Naast de wetenschappelijke bijdrage die je geleverd hebt aan dit proefschrift was het ook erg prettig om iemand in de buurt te hebben die een feilloos gevoel heeft voor het gebruik van komma's, koppeltekens, tussen-n-en en wat al niet meer.

Naast Jan wil ik **Aalzen** bedanken voor dagelijkse "hands on" begeleiding tijdens mijn onderzoek. Ik denk dat we het aantal geplande afspraken om over experimenten, data en planningen te praten op één hand kunnen tellen. Het was echter altijd mogelijk om met een vraag of een set nieuwe data bij je langs te komen en deze even te bespreken. Ook zijn er verrassend veel beslissingen gemaakt en problemen opgelost onder het genot van een kop koffie in de koffiekamer of de kantine. Ik stel je pragmatische kijk op het leven en de wetenschap erg op prijs, ook al sluit deze soms iets te goed aan op mijn pragmatische kijk op het leven. We hebben ook twee keer voor een congres de sneeuw opgezocht; een keer naar Banff voor een Keystone meeting en naar Rotterdam voor de RSV meeting. Onder het genot van een aantal glazen bier hebben we elkaar daar beter leren kennen, iets dat volgens mij de werksfeer altijd ten goede komt. Als laatst wil ik je bedanken voor je significante immunologische bijdrage aan dit proefschrift. In tegenstelling tot veel andere takken van wetenschap is de immunologie alles behalve zwart-wit. Jouw uitgebreide immunologische kennis kwam dan ook vaak van pas als ik weer eens wat te kort door de bocht ging in mijn redeneringen. In het begin vond ik dit maar wat vervelend -zwart-wit is immers veel overzichtelijker- maar naarmate mijn onderzoek vorderde kwam het besef dat het afweersysteem een bondgenoot is in het bestrijden van infectieziekten zolang je het op de juiste manier stimuleert, in tegenstelling tot een vervelende hindernis die ervoor zorgt dat dingen niet zo gaan als ze moeten.

Ik heb het altijd plezierig gevonden dat mijn onderzoek deel uitmaakte van een Top Institute Pharma consortium. Het idee van TI-Pharma is dat de academie en de industrie elkaar vinden. Dat ik dit als plezierig heb ervaren is natuurlijk voor een groot deel te danken aan de toevoeging die **Toon** heeft geleverd aan dit onderzoek. In het begin kwam je af en toe langs om de lopende zaken en experimenten te bespreken en was ik enorm onder de indruk van jouw kennis over eigenlijk alles wat met vaccins te maken had. Jouw kijk op de proeven was net even anders

dan die van Jan, Aalzen en mijzelf en ik denk dat dit samen tot een mooi evenwicht leidde. De presentaties over het werk dat jullie bij Mymetics doen om de RSV virosomen verder te ontwikkelen en klaar te maken voor klinische studies vond ik altijd erg interessant. Hierdoor heb ik jouw bezoekjes aan Groningen en onze bezoekjes aan Leiden altijd als erg motiverend ervaren.

De laatste persoon die ook een significante bijdrage heeft geleverd aan alle experimentele hoofdstukken in dit boekje is **Tjarko**. Jouw hulp in het CDL en in het lab met het verwerken van de monsters was van onschatbare waarde en daarnaast ook nog eens erg gezellig. We hebben samen de nodige muisexperimenten gedaan en het cotton rat model weer opgezet in Groningen. Vooral de cotton rats zorgden door hun "enthousiasme" voor de nodige memorabele momenten. Als ik terugdenk aan alle ELISA platen die jij doorverdund hebt door de jaren heen krijg ik nog steeds last van mijn duimen. Het was ook altijd erg prettig dat we bij elkaar in de kamer zaten. Als ik vragen had over DECs of dieren waren die in elk geval snel gesteld en beantwoord.

Performing experiments and writing a thesis is one thing, but evaluating the content of this book for its scientific merit is something else. I would like to thank **Professor Willem Van Eden, Professor Ruth Karron and Professor Henkjan Verkade** for taking the time to read the manuscript and for supplying their contributions to the final version.

While I'm typing in English, I might as well take the time now to thank **Shafique** for the experiments we performed together. I really admire the fact that you left your home country in search of a challenge and the enormous effort you put into your research. Your work on optimization of intranasal RSV vaccine candidates met my project and we worked together to see if RSV-MPLA virosomes were also suited for intranasal administration. While this did work, I am looking forward to your data on the RSV-MDP-Pam3CSK virosomes.

En dan schakelen we nu weer naar het Nederlands want dat kan **Julia** best lezen ondanks haar Duitse afkomst. Ik vond het erg leerzaam en gezellig dat jij bijna een jaar bij mij stage hebt gelopen. Je pakte alle experimenten snel op en je werkte super netjes en overzichtelijk. Je werk heeft geleid tot een mooi stage en afstudeer verslag en een aantal experimenten zijn ook in dit boekje terecht gekomen. Naast je goede werk heb je er ook voor gezorgd dat ik zelf, toch zeker een jaar lang min of meer op tijd op het lab kwam. Je was er meestal wel eerder dan ik, maar veel scheelde het niet.

Naast de mensen die op een concrete manier hebben bijgedragen aan de hoofdstukken van dit proefschrift zijn er natuurlijk nog veel meer mensen die hebben bijgedragen aan het prettige klimaat waarin het allemaal tot stand is gekomen. Na een aantal omzwervingen en kamerwisselingen besloot Jan dat de meest stabiele oplossing een kamer was met **Tjarko, Wouter, Tjalke, Natalija** en mijzelf. Vier mannen die fungeerden als regelstaven in een

kernreactor die regelmatig de “meltdown” van de uiterst volatiele Natalija voorkwamen. Ik denk dat het moeilijk is om een kamer samen te stellen met vijf mensen die minder op elkaar lijken maar deze diversiteit zorgde ervoor dat het altijd gezellig was. We hebben in elk geval veel goede gesprekken en leuke feestjes met elkaar meegemaakt. Tjalke, Wouter en Natalija zijn inmiddels net als ik vertrokken bij de Moleculaire Virologie en ik wens jullie dan ook veel succes en plezier bij jullie nieuwe werkgevers.

Dan wil ik natuurlijk nog Paul en Natalija bedanken dat zij mij tijdens mijn verdediging bij willen staan. **Paul**, we kennen elkaar ruim 12 jaar en ik kan me ons eerste gesprek nog goed herinneren. Ik denk nog vaak aan hoe de wereld er nu uit had gezien als we ons hydraulisch geveerde barconcept verder hadden ontwikkeld. Doordat jij met het briljante idee kwam om het eerste weekend van augustus in 2001 te gaan zeilen hebben we nu een gezamenlijke hobby waar we al vele jaren plezier aan beleven. **Natalija**, as was mentioned by the lady who wed you and Koen, we got of to a rough start. You thought I was arrogant and I thought the same of you. You are an acquired taste and it lasted almost the full four years of our PhD, for us to really get to know each other. I’d like to thank you for our good scientific and non-scientific discussions and of course for our dinners together. I’m sorry and proud of the fact that my birthday party is the reason you don’t drink alcohol anymore. That will be something to remember me by.

Verder wil ik ook alle mensen die de laatste jaren bij de **Moleculaire Virologie** hebben gewerkt bedanken voor de prettige samenwerking. Ondanks de enorme groei die de afdeling de laatste jaren heeft doorgemaakt bleef het een prettige plek om te werken. De labuitjes, borrels en feestjes waren altijd een erg welkome afwisseling.

Naast het werk heb ik natuurlijk ook erg veel tijd doorgebracht met goede vrienden en kennissen. Voor de nodige inspannende ontspanning kon ik altijd in klimcentrum **Bjoeks** terecht. Ik wil dan ook alle mensen die ik in Bjoeks heb leren kennen bedanken voor de mooie tijd. Ik hoop dat de traditionele BBX naar een jaar afwezigheid in 2013 zal terugkeren als nooit tevoren.

Als ik niet aan het klimmen of aan het werken was, bevond ik mij regelmatig op het water of in de kroeg met vrienden van **Za Zdarowja**. Ik ben blij dat ik aan die twee jaar Enschede zo veel goede vrienden heb overgehouden en ondanks dat iedereen nu een beetje begint te settelen, denk ik dat de collectie prachtige avonturen die we samen mee hebben gemaakt nog wel gaat uitbreiden.

De persoon die me tijdens mijn tijd in Groningen denk ik het meest heeft gezien moet toch wel **Martine** zijn. Ik kon als ik je zag altijd even over mijn werk praten en ik merkte dat ik daar veel aan had. Daarnaast wil ik je heel erg bedanken voor de leuke tijd die we samen hebben gehad en alles wat we samen gedaan hebben. Ik denk nog vaak terug aan onze tijd samen en ik weet zeker dat ik je nooit zal vergeten.

Als laatst wil ik nog mijn familie bedanken voor alle kansen en steun die ze me gegeven hebben. Ik kan gelukkig zeggen dat ik een prettige jeugd heb gehad en dit kwam zeker in de eerste plaats door **Papa, Mama en Liselot**. Pap en mam hebben me altijd alle kansen gegeven die ik nodig had en gesteund bij elke beslissing die ik nam. Jullie konden het in elk geval goed verbergen als jullie dachten dat het niet echt goed zou komen. Elke keer als ik in Almelo op bezoek kwam vroegen jullie hoe het ging met de vier experimentele hoofdstukken die ik voor mijn proefschrift nodig zou hebben. Dit zou er immers één per jaar moeten zijn. Ik bleef maar zeggen dat ik nog wel op schema lag en dat het zwaartepunt altijd ergens op het eind ligt. Uiteindelijk is het ook goed gekomen en wordt dit hoofdstuk ook weer afgesloten. Ik ben helaas niet dichter bij komen wonen, maar Den Haag is ook niet de andere kant van de wereld en ik hoop dan ook dat jullie vaak op bezoek zullen komen. Ik ben ook trots op mijn kleine zusje Liselot die er niet zo goed tegen kan dat ik haar mijn kleine zusje noem. Ik ben blij dat je je draai gevonden hebt en dat je weer op je plekje bent in Almelo. Je hebt het goed voor elkaar en je zal altijd mijn kleine zusje blijven, of je dat nou leuk vindt of niet.

Al met al heb ik een boel om dankbaar voor te zijn en dit komt niet in de laatste plaats door alle bijzondere mensen die ik de laatste jaren heb leren kennen. Het lijkt me fantastisch om met jullie allemaal contact te houden. Ik zal dan ook nog regelmatig mijn hoofd laten zien in het noorden en het oosten van het land. Daarnaast zijn jullie natuurlijk allemaal van harte welkom voor een biertje, een kop koffie of een etentje in de Hofstad.

Dank jullie wel,

Tobias

List of publications

Lipopeptide-adjuvanted respiratory syncytial virus virosomes: A safe and immunogenic non-replicating vaccine formulation. Toon Stegmann, Tobias Kamphuis, Tjarko Meijerhof, Ellen Goud, Aalzen de Haan, Jan Wilschut. *Vaccine, 2010 Aug 2;28(34): 5543-50*

Immunogenicity and protective capacity of a virosomal Respiratory Syncytial Virus vaccine adjuvanted with monophosphoryl lipid A in mice. Tobias Kamphuis, Tjarko Meijerhof, Toon Stegmann, Julia Lederhofer, Jan Wilschut, Aalzen de Haan. *PLoS One. 2012;7(5):e36812*

Nanoaggregates of micropurified lipopolysaccharide identified using dynamic 3 light scattering, zeta potential measurement, and TLR4 signaling activity. Eugenio Hardy, Tobias Kamphuis, Aleksandre Japaridze, Jan C. Wilschut, Mathias Winterhalter. *Anal. Biochemistry 2012 (in press)*

A virosomal Respiratory Syncytial Virus vaccine adjuvanted with MPLA provides protection against viral challenge without priming for enhanced disease in cotton rats. Tobias Kamphuis, Toon Stegmann, Tjarko Meijerhof, Jan Wilschut, Aalzen de Haan. *Submitted to Influenza and Other Respiratory Viruses*

Efficacy and safety of an intranasal virosomal Respiratory Syncytial Virus vaccine adjuvanted with Monophosphoryl Lipid A in mice and cotton rats. Tobias Kamphuis, Muhammad Shafique, Tjarko Meijerhof, Toon Stegmann, Jan Wilschut, Aalzen de Haan. *Submitted to Vaccine*

A virosomal Respiratory Syncytial Virus vaccine adjuvanted with Monophosphoryl Lipid A: Immunogenicity and protective efficacy in aged cotton rats. Tobias Kamphuis, Toon Stegmann, Tjarko Meijerhof, Jan Wilschut, Aalzen de Haan. *Manuscript in preparation*

References

1. Tregoning JS, Schwarze J. Respiratory viral infections in infants: causes, clinical symptoms, virology, and immunology. *Clin Microbiol Rev.* 2010 Jan.;23(1):74–98.

2. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *Lancet.* 2010 May 1;375(9725):1545–1555.

3. Thompson WW, Shay DK, Weintraub E, Brammer L, Cox N, Anderson LJ, et al. Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA.* 2003 Jan. 8;289(2):179–186.

4. Hall CB. Respiratory syncytial virus and parainfluenza virus. *N Engl J Med.* 2001 Jun. 21;344(25):1917–1928.

5. Henrickson KJ, Hoover S, Kehl KS, Hua W. National disease burden of respiratory viruses detected in children by polymerase chain reaction. *Pediatr Infect Dis J.* 2004 Jan.;23(1 Suppl):S11–8.

6. Hall CB, Long CE, Schnabel KC. Respiratory syncytial virus infections in previously healthy working adults. *Clin Infect Dis.* 2001 Sep. 15;33(6):792–796.

7. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. *N Engl J Med.* 2005 Apr. 28;352(17):1749–1759.

8. Raboni SM, Nogueira MB, Tsuchiya LRV, Takahashi GA, Pereira LA, Pasquini R, et al. Respiratory tract viral infections in bone marrow transplant patients. *Transplantation.* 2003 Jul. 15;76(1):142–146.

9. Roymans D, Koul A. Respiratory syncytial virus: a prioritized or neglected target? 2010;

10. Hacking D, Hull J. Respiratory syncytial virus--viral biology and the host response. *J Infect.* 2002 Jul.;45(1):18–24.

11. Lo M, Brazas R. Respiratory Syncytial Virus Nonstructural Proteins NS1 and NS2 Mediate Inhibition of Stat2 Expression and Alpha/Beta Interferon Responsiveness. *J Virol.* 2005;

12. Bossert B, Marozin S. Nonstructural Proteins NS1 and NS2 of Bovine Respiratory Syncytial Virus Block Activation of Interferon Regulatory Factor 3. *J Virol.* 2003;

13. Ling Z, Tran KC, Teng MN. Human respiratory syncytial virus nonstructural protein NS2 antagonizes the activation of beta interferon transcription by interacting with RIG-I. *J Virol.* 2009 Apr.;83(8):3734–3742.

14. Gan SW, Ng L, Lin X, Gong X, Torres J. Structure and ion channel activity of the human respiratory syncytial virus (hRSV) small hydrophobic protein transmembrane domain. *Protein Sci.* 2008 May;17(5):813–820.

15. Teng MN, Whitehead SS, Collins PL. Contribution of the respiratory syncytial virus G glycoprotein and its secreted and membrane-bound forms to virus replication in vitro and in vivo. *Virology.* 2001 Oct. 25;289(2):283–296.

16. Widjojoatmodjo MN, Boes J, van Bers M, van Remmerden Y, Roholl PJM, Luytjes W. A highly attenuated recombinant human respiratory syncytial virus lacking the G protein induces long-lasting protection in cotton rats. *Virol J.* 2010;7:114.

17. Johnson TR, Graham BS. Secreted respiratory syncytial virus G glycoprotein induces interleukin-5 (IL-5), IL-13, and eosinophilia by an IL-4-independent mechanism. *J Virol.* 1999 Oct.;73(10):8485–8495.

18. Shingai M, Azuma M, Ebihara T, Sasai M, Funami K, Ayata M, et al. Soluble G protein of respiratory syncytial virus inhibits Toll-like receptor 3/4-mediated IFN-beta induction. *Int Immunol.* 2008 Sep.;20(9):1169–1180.

19. Tayyari F, Marchant D, Moraes TJ, Duan W, Mastrangelo P, Hegele RG. Identification of nucleolin as a cellular receptor for human respiratory syncytial virus. *Nat Med.* 2011 Sep.;17(9):1132–1135.

20. Calder LJ, González-Reyes L, García-Barreno B, Wharton SA, Skehel JJ, Wiley DC, et al. Electron microscopy of the human respiratory syncytial virus fusion protein and complexes that it forms with monoclonal antibodies. *Virology.* 2000 May 25;271(1):122–131.

21. Srinivasakumar N, Ogra PL, Flanagan TD. Characteristics of fusion of respiratory syncytial virus with HEp-2 cells as measured by R18 fluorescence dequenching assay. *J Virol.* 1991 Aug.;65(8):4063–4069.

22. Fearns R. Role of the M2-1 Transcription Antitermination Protein of Respiratory Syncytial Virus in Sequential Transcription. *J Virol.* 1999;

23. Cheng X, Park H, Zhou H, Jin H. Overexpression of the M2-2 protein of respiratory syncytial virus inhibits viral replication. *J Virol.* 2005 Nov.;79(22):13943–13952.

24. Stec DS, Hill MG, Collins PL. Sequence analysis of the polymerase L gene of human respiratory syncytial virus and predicted phylogeny of nonsegmented negative-strand viruses. *Virology.* 1991 Jul.;183(1):273–287.

25. Krilov LR. Respiratory syncytial virus: Update on infection, treatment, and prevention. *Curr Infect Dis Rep.* 2001 Jun.;3(3):242–246.

26. Backovic M, Rey FA. Virus entry: old viruses, new receptors. *Curr Opin Virol.* 2012 Feb.;2(1):4–13.

27. Bermingham A, Collins PL. The M2-2 protein of human respiratory syncytial virus is a regulatory factor involved in the balance between RNA replication and transcription. *Proc Natl Acad Sci USA.* 1999 Sep. 28;96(20):11259–11264.

28. Brown G, Jeffree CE, McDonald T, Rixon HWM, Aitken JD, Sugrue RJ. Analysis of the interaction between respiratory syncytial virus and lipid-rafts in Hep2 cells during infection. *Virology.* 2004 Oct. 1;327(2):175–185.

29. Pastey MK, Gower TL, Spearman PW, Crowe JE, Graham BS. A RhoA-derived peptide inhibits syncytium formation induced by respiratory syncytial virus and parainfluenza virus type 3. *Nat Med.* 2000 Jan.;6(1):35–40.

30. Sattentau Q. Avoiding the void: cell-to-cell spread of human viruses. *Nat Rev Microbiol.* 2008 Nov.;6(11):815–826.

31. Hall C. Respiratory Syncytial Virus and Parainfluenza Virus — NEJM. *new England journal of medicine.* 2001;

32. González PA, Bueno SM, Carreño LJ, Riedel CA, Kalergis AM. Respiratory syncytial virus infection and immunity. *Rev Med Virol.* 2012 Jan. 31;

33. Handforth J, Friedland JS, Sharland M. Basic epidemiology and immunopathology of RSV in children. *Paediatr Respir Rev.* 2000 Sep.;1(3):210–214.

34. Miyairi I, DeVincenzo JP. Human genetic factors and respiratory syncytial virus disease severity. *Clin Microbiol Rev.* 2008 Oct.;21(4):686–703.

35. Janssen R, Bont L, Siezen CLE, Hodemaekers HM, Ermers MJ, Doornbos G, et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis is predominantly associated with innate immune genes. *J Infect Dis.* 2007 Sep. 15;196(6):826–834.

36. Siezen CLE, Bont L, Hodemaekers HM, Ermers MJ, Doornbos G, Van't Slot R, et al. Genetic susceptibility to respiratory syncytial virus bronchiolitis in preterm children is associated with airway remodeling genes and innate immune genes. *Pediatr Infect Dis J.* 2009 Apr.;28(4):333–335.

37. Rudd BD, Luker GD, Luker KE, Peebles RS, Lukacs NW. Type I interferon regulates respiratory virus infected dendritic cell maturation and cytokine production. *Viral Immunol.* 2007 Dec. 1;20(4):531–540.

38. Biron CA. Interferons alpha and beta as immune regulators--a new look. *Immunity.* 2001 Jun.;14(6):661–664.

39. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. *Nature.* 1998 Mar. 19;392(6673):245–252.

40. Netea MG, Van der Meer JWM, Sutmuller RP, Adema GJ, Kullberg B-J. From the Th1/Th2 Paradigm towards a Toll-Like Receptor/T-Helper Bias. *Antimicrobial Agents and Chemotherapy.* 2005 Oct. 1;49(10):3991.

41. Becker Y. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy--a review. *Virus Genes.* 2006 Oct.;33(2):235–252.

42. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol.* 2000 Nov.;1(5):398–

401.

43. Haynes LM, Moore DD, Kurt-Jones EA, Finberg RW, Anderson LJ, Tripp RA. Involvement of toll-like receptor 4 in innate immunity to respiratory syncytial virus. *J Virol.* 2001 Nov.;75(22):10730–10737.

44. Puthothu B, Forster J, Heinzmann A, Krueger M. TLR-4 and CD14 polymorphisms in respiratory syncytial virus associated disease. *Dis Markers.* 2006;22(5-6):303–308.

45. Patel JA, Kunimoto M, Sim TC, Garofalo R, Eliott T, Baron S, et al. Interleukin-1 alpha mediates the enhanced expression of intercellular adhesion molecule-1 in pulmonary epithelial cells infected with respiratory syncytial virus. *American Journal of Respiratory Cell and Molecular Biology.* 1995 Nov.;13(5):602–609.

46. Noah TL, Henderson FW, Wortman IA, Devlin RB, Handy J, Koren HS, et al. Nasal cytokine production in viral acute upper respiratory infection of childhood. *J Infect Dis.* 1995 Mar.;171(3):584–592.

47. Becker S, Reed W, Henderson FW, Noah TL. RSV infection of human airway epithelial cells causes production of the beta-chemokine RANTES. *Am J Physiol.* 1997 Mar.;272(3 Pt 1):L512–20.

48. Kawai T, Akira S. Antiviral signaling through pattern recognition receptors. *J Biochem.* 2007 Feb.;141(2):137–145.

49. Tripp RA. Pathogenesis of respiratory syncytial virus infection. *Viral Immunol.* 2004 Jan. 1;17(2):165–181.

50. Hall CB, Powell KR, MacDonald NE, Gala CL, Menegus ME, Suffin SC, et al. Respiratory syncytial viral infection in children with compromised immune function. *N Engl J Med.* 1986 Jul. 10;315(2):77–81.

51. Nicholas JA, Rubino KL, Levely ME, Adams EG, Collins PL. Cytolytic T-lymphocyte responses to respiratory syncytial virus: effector cell phenotype and target proteins. *J Virol.* 1990 Sep.;64(9):4232–4241.

52. Boelen A, Kwakkel J, Barends M, de Rond L, Dormans J, Kimman T. Effect of lack of Interleukin-4, Interleukin-12, Interleukin-18, or the Interferon-gamma receptor on virus replication, cytokine response, and lung pathology during respiratory syncytial virus infection in mice. *J Med Virol.* 2002 Apr.;66(4):552–560.

53. Crowe JE. Respiratory syncytial virus vaccine development. *Vaccine.* 2001 Oct. 15;20 Suppl 1:S32–7.

54. Holberg CJ, Wright AL, Martinez FD, Ray CG, Taussig LM, Lebowitz MD. Risk factors for respiratory syncytial virus-associated lower respiratory illnesses in the first year of life. *Am J Epidemiol.* 1991 Jun. 1;133(11):1135–1151.

55. Groothuis JR, Hoopes JM, Hemming VG. Prevention of serious respiratory syncytial virus-related illness. II: Immunoprophylaxis. *Adv Ther.* 2011 Feb.;28(2):110–125.

56. Welliver RC, Kaul TN, Putnam TI, Sun M, Riddlesberger K, Ogra PL. The antibody response to primary and secondary infection with respiratory syncytial virus: kinetics of class-specific responses. *J Pediatr.* 1980 May;96(5):808–813.

57. Welliver RC, Kaul TN, Ogra PL. The appearance of cell-bound IgE in respiratory-tract epithelium after respiratory-syncytial-virus infection. *N Engl J Med.* 1980 Nov. 20;303(21):1198–1202.

58. Welliver RC, Sun M, Rinaldo D, Ogra PL. Predictive value of respiratory syncytial virus-specific IgE responses for recurrent wheezing following bronchiolitis. *J Pediatr.* 1986 Nov.;109(5):776–780.

59. Falsey AR, Singh HK, Walsh EE. Serum antibody decay in adults following natural respiratory syncytial virus infection. *J Med Virol.* 2006 Nov.;78(11):1493–1497.

60. Group TI-RS. Palivizumab, a humanized respiratory syncytial virus monoclonal antibody, reduces hospitalization from respiratory syncytial virus infection in high-risk infants. *Pediatrics.* 1998 Sep.;102(3 Pt 1):531–537.

61. Feltes TF, Cabalka AK, Meissner HC, Piazza FM, Carlin DA, Top FH, et al. Palivizumab prophylaxis reduces hospitalization due to respiratory syncytial virus in young children with hemodynamically significant congenital heart disease. *J Pediatr.* 2003 Oct.;143(4):532–540.

62. Hu J, Robinson JL. Treatment of respiratory syncytial virus with palivizumab: a systematic re-

view. *World J Pediatr.* 2010 Nov.;6(4):296–300.

63. Ventre K, Randolph AG. Ribavirin for respiratory syncytial virus infection of the lower respiratory tract in infants and young children. *Cochrane Database Syst Rev.* 2007;(1):CD000181.

64. Falsey AR, Walsh EE. Respiratory syncytial virus infection in adults. *Clin Microbiol Rev.* 2000 Jul.;13(3):371–384.

65. Walsh EE. Respiratory syncytial virus infection in adults. *Semin Respir Crit Care Med.* 2011 Aug.;32(4):423–432.

66. Nathanson N, Kew OM. From emergence to eradication: the epidemiology of poliomyelitis deconstructed. *Am J Epidemiol.* 2010 Dec. 1;172(11):1213–1229.

67. Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am J Epidemiol.* 1969 Apr.;89(4):405–421.

68. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am J Epidemiol.* 1969 Apr.;89(4):422–434.

69. Chin J, Magoffin RL, Shearer LA, Schieble JH, Lennette EH. Field evaluation of a respiratory syncytial virus vaccine and a trivalent parainfluenza virus vaccine in a pediatric population. *Am J Epidemiol.* 1969 Apr.;89(4):449–463.

70. Fulginiti VA, Eller JJ, Sieber OF, Joyner JW, Minamitani M, Meiklejohn G. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. *Am J Epidemiol.* 1969 Apr.;89(4):435–448.

71. Nader P, Horwitz M. Atypical exanthem following exposure to natural measles: eleven cases in children previously inoculated with killed vaccine. *J Pediatr.* 1968;

72. Murphy BR, Prince GA, Walsh EE, Kim HW, Parrott RH, Hemming VG, et al. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. *J Clin Microbiol.* 1986 Aug.;24(2):197–202.

73. Murphy BR, Walsh EE. Formalin-inactivated respiratory syncytial virus vaccine induces antibodies to the fusion glycoprotein that are deficient in fusion-inhibiting activity. *J Clin Microbiol.* 1988 Aug.;26(8):1595–1597.

74. Polack FP, Teng MN, Collins PL, Prince GA, Exner M, Regele H, et al. A role for immune complexes in enhanced respiratory syncytial virus disease. *J Exp Med.* 2002 Sep. 16;196(6):859–865.

75. Polack FP, Hoffman SJ, Crujeiras G, Griffin DE. A role for nonprotective complement-fixing antibodies with low avidity for measles virus in atypical measles. *Nat Med.* 2003 Sep.;9(9):1209–1213.

76. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. *Nat Med.* 2009 Jan.;15(1):34–41.

77. Waris ME, Tsou C, Erdman DD, Zaki SR, Anderson LJ. Respiratory synctial virus infection in BALB/c mice previously immunized with formalin-inactivated virus induces enhanced pulmonary inflammatory response with a predominant Th2-like cytokine pattern. *J Virol.* 1996 May;70(5):2852–2860.

78. Boukhvalova MS, Prince GA, Soroush L, Harrigan DC, Vogel SN, Blanco JCG. The TLR4 agonist, monophosphoryl lipid A, attenuates the cytokine storm associated with respiratory syncytial virus vaccine-enhanced disease. *Vaccine.* 2006 Jun. 5;24(23):5027–5035.

79. Tang YW, Graham BS. Anti-IL-4 treatment at immunization modulates cytokine expression, reduces illness, and increases cytotoxic T lymphocyte activity in mice challenged with respiratory syncytial virus. *J Clin Invest.* 1994 Nov.;94(5):1953–1958.

80. Moghaddam A, Olszewska W, Wang B, Tregoning JS, Helson R, Sattentau QJ, et al. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. *Nat Med.* 2006 Jul. 23;12(8):905–907.

81. Kruijsen D, Schijf MA, Lukens MV, van Uden NO, Kimpen JL, Coenjaerts FE, et al. Local innate and adaptive immune responses regulate inflammatory cell influx into the lungs after vaccination with formalin inactivated RSV. *Vaccine.* 2011 Mar. 24;29(15):2730–2741.

82. Neuzil KM, Johnson JE, Tang YW, Prieels JP, Slaoui M, Gar N, et al. Adjuvants influence the quantitative and qualitative immune response in BALB/c mice immunized with respiratory syncytial virus FG subunit vaccine. *Vaccine.* 1997 Apr.;15(5):525–532.

83. Kool M, Soullié T, van Nimwegen M, Willart MAM, Muskens F, Jung S, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. *J Exp Med.* 2008 Apr. 14;205(4):869–882.

84. Graham BS. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. *Immunol Rev.* 2011 Jan.;239(1):149–166.

85. Crowe JE, Bui PT, London WT, Davis AR, Hung PP, Chanock RM, et al. Satisfactorily attenuated and protective mutants derived from a partially attenuated cold-passaged respiratory syncytial virus mutant by introduction of additional attenuating mutations during chemical mutagenesis. *Vaccine.* 1994 Jun.;12(8):691–699.

86. Karron RA, Wright PF, Crowe JE, Clements-Mann ML, Thompson J, Makhene M, et al. Evaluation of two live, cold-passaged, temperature-sensitive respiratory syncytial virus vaccines in chimpanzees and in human adults, infants, and children. *J Infect Dis.* 1997 Dec.;176(6):1428–1436.

87. Wright PF, Karron RA, Belshe RB, Thompson J, Crowe JE, Boyce TG, et al. Evaluation of a Live, Cold-Passaged, Temperature-Sensitive, Respiratory Syncytial Virus Vaccine Candidate in Infancy. 2000;

88. Karron RA, Wright PF, Belshe RB, Thumar B, Casey R, Newman F, et al. Identification of a recombinant live attenuated respiratory syncytial virus vaccine candidate that is highly attenuated in infants. *J Infect Dis.* 2005 Apr. 1;191(7):1093–1104.

89. Piedra PA, Cron SG, Jewell A, Hamblett N, McBride R, Palacio MA, et al. Immunogenicity of a new purified fusion protein vaccine to respiratory syncytial virus: a multi-center trial in children with cystic fibrosis. *Vaccine.* 2003 Jun. 2;21(19-20):2448–2460.

90. Munoz FM, Piedra PA, Glezen WP. Safety and immunogenicity of respiratory syncytial virus purified fusion protein-2 vaccine in pregnant women. *Vaccine.* 2003 Jul. 28;21(24):3465–3467.

91. Falsey AR, Walsh EE. Safety and immunogenicity of a respiratory syncytial virus subunit vaccine (PFP-2) in ambulatory adults over age 60. *Vaccine.* 1996 Sep.;14(13):1214–1218.

92. Durbin AP, Karron RA. Progress in the development of respiratory syncytial virus and parainfluenza virus vaccines. *Clin Infect Dis.* 2003 Dec. 15;37(12):1668–1677.

93. Pantua HD, McGinnes LW, Peeples ME, Morrison TG. Requirements for the Assembly and Release of Newcastle Disease Virus-Like Particles. 2006;

94. Power UF, Plotnicky-Gilquin H, Huss T, Robert A, Trudel M, Ståhl S, et al. Induction of protective immunity in rodents by vaccination with a prokaryotically expressed recombinant fusion protein containing a respiratory syncytial virus G protein fragment. *Virology.* 1997 Apr. 14;230(2):155–166.

95. Power UF, Nguyen TN, Rietveld E, de Swart RL, Groen J, Osterhaus AD, et al. Safety and immunogenicity of a novel recombinant subunit respiratory syncytial virus vaccine (BBG2Na) in healthy young adults. *J Infect Dis.* 2001 Dec. 1;184(11):1456–1460.

96. de Waal L, Power U, Yüksel S, van Amerongen G. Evaluation of BBG2Na in infant macaques: specific immune responses after vaccination and RSV challenge. *Vaccine.* 2004;

97. Lindell DM, Morris SB, White MP, Kallal LE, Lundy PK, Hamouda T, et al. A novel inactivated intranasal respiratory syncytial virus vaccine promotes viral clearance without Th2 associated vaccine-enhanced disease. *PLoS ONE.* 2011 Jul. 15;6(7):e21823.

98. Swanson KA, Settembre EC, Shaw CA, Dey AK, Rappuoli R, Mandl CW, et al. Structural basis for immunization with postfusion respiratory syncytial virus fusion F glycoprotein (RSV F) to elicit high neutralizing antibody titers. *Proc Natl Acad Sci USA.* 2011 Jun. 7;108(23):9619–9624.

99. Tang RS, MacPhail M, Schickli JH, Kaur J, Rob-

inson CL, Lawlor HA, et al. Parainfluenza Virus Type 3 Expressing the Native or Soluble Fusion (F) Protein of Respiratory Syncytial Virus (RSV) Confers Protection from RSV Infection in African Green Monkeys. 2004;

100. Gomez M, Mufson MA, Dubovsky F, Knightly C, Zeng W, Losonsky G. Phase-I study MEDI-534, of a live, attenuated intranasal vaccine against respiratory syncytial virus and parainfluenza-3 virus in seropositive children. *Pediatr Infect Dis J.* 2009 Jul.;28(7):655–658.

101. Tang R, Spaete R, Thompson M, MacPhail M. Development of a PIV-vectored RSV vaccine: Preclinical evaluation of safety, toxicity, and enhanced disease and initial clinical testing in healthy adults. *Vaccine.* 2008;

102. Bernstein DI, Malkin E, Abughali N, Falloon J, Yi T, Dubovsky F, et al. Phase 1 Study of the Safety and Immunogenicity of a Live, Attenuated Respiratory Syncytial Virus and Parainfluenza Virus Type 3 Vaccine in Seronegative Children. *Pediatr Infect Dis J.* 2012 Feb.;31(2):109–114.

103. Mok H, Lee S, Utley TJ, Shepherd BE, Polosukhin VV, Collier ML, et al. Venezuelan equine encephalitis virus replicon particles encoding respiratory syncytial virus surface glycoproteins induce protective mucosal responses in mice and cotton rats. *J Virol.* 2007 Dec.;81(24):13710–13722.

104. Almeida JD, Edwards DC, Brand CM, Heath TD. Formation of virosomes from influenza subunits and liposomes. *Lancet.* 1975 Nov. 8;2(7941):899–901.

105. Cusi M. Review Applications of Influenza Virosomes as a Delivery System. *Hum Vaccin.* 2006;

106. Huckriede A, Bungener L, Stegmann T, Daemen T, Medema J, Palache AM, et al. The virosome concept for influenza vaccines. *Vaccine.* 2005 Jul. 8;23 Suppl 1:S26–38.

107. Glück R. Adjuvant activity of immunopotentiating reconstituted influenza virosomes (IRIVs). *Vaccine.* 1999;

108. Usonis V, Bakasénas V, Valentelis R, Katiliene G, Vidzeniene D, Herzog C. Antibody titres after primary and booster vaccination of infants and young children with a virosomal hepatitis A vaccine (Epaxal). *Vaccine.* 2003 Nov. 7;21(31):4588–4592.

109. Glück R, Mischler R, Brantschen S, Just M, Althaus B, Cryz SJ Jr. Immunopotentiating reconstituted influenza virus virosome vaccine delivery system for immunization against hepatitis A. *J Clin Invest.* 1992 Dec. 1;90(6):2491–2495.

110. Stegmann T, Morselt HW, Booy FP, van Breemen JF, Scherphof G, Wilschut J. Functional reconstitution of influenza virus envelopes. *EMBO J.* 1987 Sep.;6(9):2651–2659.

111. de Jonge J, Schoen P, Veer ter W, Stegmann T, Wilschut J, Huckriede A. Use of a dialyzable short-chain phospholipid for efficient solubilization and reconstitution of influenza virus envelopes. *Bba-Biomembranes.* 2006;1758(4):527–536.

112. van Duin D, Medzhitov R, Shaw AC. Triggering TLR signaling in vaccination. *Trends Immunol.* 2006 Jan.;27(1):49–55.

113. Takeda K, Akira S. TLR signaling pathways. *Semin Immunol.* 2004 Feb. 1;16(1):3–9.

114. Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R. Toll-like receptors control activation of adaptive immune responses. *Nat Immunol.* 2001 Oct. 1;2(10):947–950.

115. Duthie MS, Windish HP, Fox CB, Reed SG. Use of defined TLR ligands as adjuvants within human vaccines. *Immunol Rev.* 2011 Jan.;239(1):178–196.

116. THERIEN H, SHAHUM E. Importance of Physical Association Between Antigen and Liposomes in Liposomes Adjuvanticity. *Immunol Lett.* 1989;22(4):253–258.

117. Kovacs-Simon A, Titball RW, Michell SL. Lipoproteins of bacterial pathogens. *Infect Immun.* 2011 Feb.;79(2):548–561.

118. Melchers F, Braun V, Galanos C. The lipoprotein of the outer membrane of Escherichia coli: a B-lymphocyte mitogen. *J Exp Med.* 1975 Aug. 1;142(2):473–482.

119. Norgard MV, Arndt LL, Akins DR, Curetty LL, Harrich DA, Radolf JD. Activation of human monocytic cells by Treponema pallidum and Borrelia burgdorferi lipoproteins and synthetic lipopeptides proceeds via a pathway distinct from that of lipopolysaccharide but involves the transcrip-

tional activator NF-kappa B. *Infect Immun.* 1996 Sep.;64(9):3845–3852.

120. Kreutz M, Ackermann U, Hauschildt S, Krause SW, Riedel D, Bessler W, et al. A comparative analysis of cytokine production and tolerance induction by bacterial lipopeptides, lipopolysaccharides and Staphyloccous aureus in human monocytes. *Immunology.* 1997 Nov.;92(3):396–401.

121. Wiesmüller K, Bessler W, Jung G. Synthesis of the Mitogenic S-[2,3-Bis(Palmitoyloxy) Propyl]-N-Palmitoylpentapeptide From Escherichia-Coli Lipoprotein. *H-S Z Physiol Chem.* 1983;364(5):593–606.

122. Bessler WG, Cox M, Lex A, Suhr B, Wiesmüller KH, Jung G. Synthetic lipopeptide analogs of bacterial lipoprotein are potent polyclonal activators for murine B lymphocytes. *J Immunol.* 1985 Sep.;135(3):1900–1905.

123. Hioe CE, Qiu H, Chend PD, Bian Z, Li ML, Li J, et al. Comparison of adjuvant formulations for cytotoxic T cell induction using synthetic peptides. *Vaccine.* 1996 Apr.;14(5):412–418.

124. Aliprantis A, Yang R, Mark M, Suggett S, Devaux B, Radolf J, et al. Cell activation and apoptosis by bacterial lipoproteins through toll-like receptor-2. *Science.* 1999;285(5428):736–739.

125. Poltorak A, He X, Smirnova I, Liu M, Van Huffel C, Du X, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: Mutations in Tlr4 gene. *Science.* 1998;282(5396):2085–2088.

126. Ulrich JT, Myers KR. Monophosphoryl lipid A as an adjuvant. Past experiences and new directions. *Pharm Biotechnol.* 1995;6:495–524.

127. Garçon N, Segal L, Tavares F, van Mechelen M. The safety evaluation of adjuvants during vaccine development: the AS04 experience. *Vaccine.* 2011 Jun. 15;29(27):4453–4459.

128. Garçon N, Wettendorff M, van Mechelen M. Role of AS04 in human papillomavirus vaccine: mode of action and clinical profile. *Expert Opin Biol Ther.* 2011 May;11(5):667–677.

129. Prince GA, Denamur F, Deschamps M, Garçon N, Prieels JP, Slaoui M, et al. Monophosphoryl lipid A adjuvant reverses a principal histologic parameter of formalin-inactivated respiratory syncytial virus vaccine-induced disease. *Vaccine.* 2001 Feb. 28;19(15-16):2048–2054.

130. Domachowske JB, Bonville CA, Rosenberg HF. Animal models for studying respiratory syncytial virus infection and its long term effects on lung function. *Pediatr Infect Dis J.* 2004 Nov.;23(11 Suppl):S228–34.

131. Crowe J, BUI P, SIBER G, ELKINS W, CHANOCK R, MURPHY B. Cold Passaged, Temperature-Sensitive Mutants of Human Respiratory Syncytial Virus (Rsv) Are Highly Attenuated, Immunogenic, and Protective in Seronegative Chimpanzees, Even When Rsv Antibodies Are Infused Shortly Before Immunization. *Vaccine.* 1995;13(9):847–855.

132. Tang R, MacPhail M, Schickli J, Kaur J, Lawlor H, Guzzetta J, et al. Parainfluenza virus type 3 expressing the native or soluble fusion (F) protein of respiratory syncytial virus (RSV) confers protection from RSV infection in African green monkeys. *J Virol.* 2004;78(20):11198–11207.

133. KAKUK T, SOIKE K, BRIDEAU R, ZAYA R, COLE S, ZHANG J, et al. A Human Respiratory Syncytial Virus (Rsv) Primate Model of Enhanced Pulmonary Pathology Induced with a Formalin-Inactivated Rsv Vaccine but Not a Recombinant Fg Subunit Vaccine. *J Infect Dis.* 1993;167(3):553–561.

134. Bem RA, Domachowske JB, Rosenberg HF. Animal models of human respiratory syncytial virus disease. *Am J Physiol Lung Cell Mol Physiol.* 2011 May 13;

135. Blount RE, Morris JA, Savage RE. Recovery of cytopathogenic agent from chimpanzees with coryza. *Proc Soc Exp Biol Med.* 1956 Jul.;92(3):544–549.

136. Graham BS. Biological challenges and technological opportunities for respiratory syncytial virus vaccine development. *Immunol Rev.* 2010 Dec. 28;239(1):149–166.

137. Connors M, Giese NA, Kulkarni AB, Firestone CY, Morse HC, Murphy BR. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. *J Virol.* 1994 Aug.;68(8):5321–5325.

138. Connors M, Kulkarni AB, Firestone CY, Holmes KL, Morse HC, Sotnikov AV, et al. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. *J Virol.* 1992 Dec.;66(12):7444–7451.

139. Prince GA, Jenson AB, Horswood RL, Camargo E, Chanock RM. The pathogenesis of respiratory syncytial virus infection in cotton rats. *Am J Pathol.* 1978 Dec.;93(3):771–791.

140. Prince GA, Jenson AB, Hemming VG, Murphy BR, Walsh EE, Horswood RL, et al. Enhancement of respiratory syncytial virus pulmonary pathology in cotton rats by prior intramuscular inoculation of formalin-inactiva ted virus. *J Virol.* 1986 Mar.;57(3):721–728.

141. Boukhvalova MS, Prince GA, Blanco JCG. The cotton rat model of respiratory viral infections. *Biologicals.* 2009 Jun.;37(3):152–159.

142. van Drunen Littel-van den Hurk S, Mapletoft JW, Arsic N, Kovacs-Nolan J. Immunopathology of RSV infection: prospects for developing vaccines without this complication. *Rev Med Virol.* 2007 Jan.;17(1):5–34.

143. Falsey A, Hennessey P. Respiratory Syncytial Virus Infection in Elderly and High-Risk Adults. *new England journal of medicine.* 2005;

144. Murphy BR, Sotnikov AV, Lawrence LA, Banks SM, Prince GA. Enhanced pulmonary histopathology is observed in cotton rats immunized with formalin-inactivated respiratory syncytial virus (RSV) or purified F glycoprotein and challenged with RSV 3-6 months after immunization. *Vaccine.* 1990 Oct.;8(5):497–502.

145. Daemen T, de Mare A, Bungener L, de Jonge J, Huckriede A, Wilschut J. Virosomes for antigen and DNA delivery. *Adv Drug Deliv Rev.* 2005 Jan. 10;57(3):451–463.

146. Dijkstra J, Bron R, Wilschut J, de Haan A, Ryan JL. Activation of murine lymphocytes by lipopolysaccharide incorporated in fusogenic, reconstituted influenza virus envelopes (virosomes). *J Immunol.* 1996 Aug. 1;157(3):1028–1036.

147. de Vries JJC, Bungener L, Veer ter W, van Alphen L, van der Ley P, Wilschut J, et al. Incorporation of LpxL1, a detoxified lipopolysaccharide adjuvant, in influenza H5N1 virosomes increases vaccine immunogenicity. *Vaccine.* 2009 Feb. 5;27(6):947–955.

148. Cusi MG, Zurbriggen R, Correale P, Valassina M, Terrosi C, Pergola L, et al. Influenza virosomes are an efficient delivery system for respiratory syncytial virus-F antigen inducing humoral and cell-mediated immunity. *Vaccine.* 2002 Oct. 4;20(29-30):3436–3442.

149. Bessler WG, Heinevetter L, Wiesmüller KH, Jung G, Baier W, Huber M, et al. Bacterial cell wall components as immunomodulators--I. Lipopeptides as adjuvants for parenteral and oral immunization. *Int J Immunopharmacol.* 1997 Sep.;19(9-10):547–550.

150. Ponimaskin E, Bareesel KK, Markgraf K, Reszka R, Lehmann K, Gelderblom HR, et al. Sendai virosomes revisited: reconstitution with exogenous lipids leads to potent vehicles for gene transfer. *Virology.* 2000 Apr. 10;269(2):391–403.

151. Moore ML, Peebles RS. Respiratory syncytial virus disease mechanisms implicated by human, animal model, and in vitro data facilitate vaccine strategies and new therapeutics. *Pharmacol Ther.* 2006 Nov. 1;112(2):405–424.

152. Connors M, Collins PL, Firestone CY, Sotnikov AV, Waitze A, Davis AR, et al. Cotton rats previously immunized with a chimeric RSV FG glycoprotein develop enhanced pulmonary pathology when infected with RSV, a phenomenon not encountered following immunization with vaccinia--RSV recombinants or RSV. *Vaccine.* 1992;10(7):475–484.

153. Falsey AR, Walsh EE, Capellan J, Gravenstein S, Zambon M, Yau E, et al. Comparison of the safety and immunogenicity of 2 respiratory syncytial virus (rsv) vaccines--nonadjuvanted vaccine or vaccine adjuvanted with alum--given concomitantly with influenza vaccine to high-risk elderly individuals. *J Infect Dis.* 2008 Nov. 1;198(9):1317–1326.

154. Sakurai H, Williamson RA, Crowe JE, Beeler JA, Poignard P, Bastidas RB, et al. Human antibody responses to mature and immature forms of viral envelope in respiratory syncytial virus infection: significance for subunit vaccines. *J Virol.* 1999 Apr. 1;73(4):2956–2962.

155. Johnson PR, Olmsted RA, Prince GA, Murphy BR, Alling DW, Walsh EE, et al. Antigenic relatedness

between glycoproteins of human respiratory syncytial virus subgroups A and B: evaluation of the contributions of F and G glycoproteins to immunity. *J Virol.* 1987 Oct. 1;61(10):3163–3166.

156. Kessi J, Poirée JC, Wehrli E, Bachofen R, Semenza G, Hauser H. Short-chain phosphatidylcholines as superior detergents in solubilizing membrane proteins and preserving biological activity. *Biochemistry.* 1994 Sep. 6;33(35):10825–10836.

157. Shivanna BD, Rowe ES. Preservation of the native structure and function of Ca2+-ATPase from sarcoplasmic reticulum: solubilization and reconstitution by new short-chain phospholipid detergent 1,2-diheptanoyl-sn-phosphatidylcholine. *Biochem J.* 1997 Jul. 15;325 (Pt 2):533–542.

158. Langley JM, Sales V, McGeer A, Guasparini R, Predy G, Meekison W, et al. A dose-ranging study of a subunit Respiratory Syncytial Virus subtype A vaccine with and without aluminum phosphate adjuvantation in adults > or =65 years of age. *Vaccine.* 2009 Sep. 25;27(42):5913–5919.

159. Gupta CK, Leszczynski J, Gupta RK, Siber GR. Stabilization of respiratory syncytial virus (RSV) against thermal inactivation and freeze-thaw cycles for development and control of RSV vaccines and immune globulin. *Vaccine.* 1996 Oct. 1;14(15):1417–1420.

160. Böttcher C, van Gent C, Pries C. A rapid and sensitive sub-micro phosphorus determination. *Anal Chim Acta.* 1961;24:203–204.

161. Falsey AR, Walsh EE. Viral pneumonia in older adults. *Clin Infect Dis.* 2006 Feb. 15;42(4):518–524.

162. Hampp C, Kauf TL, Saidi AS, Winterstein AG. Cost-effectiveness of Respiratory Syncytial Virus Prophylaxis in Various Indications. *Arch Pediatr Adolesc Med.* 2011 May 5;

163. Polack FP, Karron RA. The future of respiratory syncytial virus vaccine development. *Pediatr Infect Dis J.* 2004 Jan. 1;23(1 Suppl):S65–73.

164. Falsey AR. Respiratory syncytial virus infection in adults. *Semin Respir Crit Care Med.* 2007 Apr.;28(2):171–181.

165. KIM H, CANCHOLA J. Respiratory syncytial virus disease in infants despite prior administra- tion of antigenic inactivated vaccine. *American Journal of ….* 1969;

166. Graham BS, Henderson GS, Tang YW, Lu X, Neuzil KM, Colley DG. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. *J Immunol.* 1993 Aug. 15;151(4):2032–2040.

167. Guy B. The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol.* 2007 Jul. 1;5(7):505–517.

168. Stegmann T, Kamphuis T, Meijerhof T, Goud E, de Haan A, Wilschut J. Lipopeptide-adjuvanted respiratory syncytial virus virosomes: A safe and immunogenic non-replicating vaccine formulation. *Vaccine.* 2010 Aug. 2;28(34):5543–5550.

169. Casella CR, Mitchell TC. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cell Mol Life Sci.* 2008 Oct.;65(20):3231–3240.

170. Johnson RS, Her GR, Grabarek J, Hawiger J, Reinhold VN. Structural characterization of monophosphoryl lipid A homologs obtained from Salmonella minnesota Re595 lipopolysaccharide. *J Biol Chem.* 1990 May 15;265(14):8108–8116.

171. Crommelin D, Stolk P, Besançon L, Shah V, Midha K, Leufkens H. Pharmaceutical sciences in 2020. *Nat Rev Drug Discov.* 2010 Feb.;9(2):99–100.

172. Akira S, Takeda K. Toll-like receptor signalling. *Nat Rev Immunol.* 2004 Jul.;4(7):499–511.

173. Guy B. The perfect mix: recent progress in adjuvant research. *Nat Rev Microbiol.* 2007 Jul.;5(7):505–517.

174. Didierlaurent AM, Morel S, Lockman L, Giannini SL, Bisteau M, Carlsen H, et al. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J Immunol.* 2009 Nov. 15;183(10):6186–6197.

175. Welliver RC, Kaul TN, Sun M, Ogra PL. Defective regulation of immune responses in respiratory syncytial virus infection. *J Immunol.* 1984 Oct.;133(4):1925–1930.

176. Welliver RC, Wong DT, Sun M, Middleton E,

Vaughan RS, Ogra PL. The development of respiratory syncytial virus-specific IgE and the release of histamine in nasopharyngeal secretions after infection. *N Engl J Med.* 1981 Oct. 8;305(15):841–846.

177. Swanson CL, Wilson TJ, Strauch P, Colonna M, Pelanda R, Torres RM. Type I IFN enhances follicular B cell contribution to the T cell-independent antibody response. *J Exp Med.* 2010 Jul. 5;207(7):1485–1500.

178. Nimmerjahn F, Ravetch JV. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science.* 2005 Dec. 2;310(5753):1510–1512.

179. Coutelier JP, van der Logt JT, Heessen FW, Warnier G, Van Snick J. IgG2a restriction of murine antibodies elicited by viral infections. *J Exp Med.* 1987 Jan. 1;165(1):64–69.

180. Heer AK, Shamshiev A, Donda A, Uematsu S, Akira S, Kopf M, et al. TLR signaling fine-tunes anti-influenza B cell responses without regulating effector T cell responses. *J Immunol.* 2007 Feb. 15;178(4):2182–2191.

181. McLellan JS, Chen M, Kim A, Yang Y, Graham BS, Kwong PD. Structural basis of respiratory syncytial virus neutralization by motavizumab. *Nat Struct Mol Biol.* 2010 Feb.;17(2):248–250.

182. Budimir N, Meijerhof T, Wilschut J, Huckriede A, de Haan A. The role of membrane fusion activity of a whole inactivated influenza virus vaccine in (re) activation of influenza-specific cytotoxic T lymphocytes. *Vaccine.* 2010 Dec. 6;28(52):8280–8287.

183. Prince GA, Curtis SJ, Yim KC, Porter DD. Vaccine-enhanced respiratory syncytial virus disease in cotton rats following immunization with Lot 100 or a newly prepared reference vaccine. *J Gen Virol.* 2001 Dec. 1;82(Pt 12):2881–2888.

184. Meyer T, Stockfleth E. Clinical investigations of Toll-like receptor agonists. *Expert Opin Investig Drugs.* 2008 Jul.;17(7):1051–1065.

185. Kamphuis T, Meijerhof T, Stegmann T, Lederhofer J, Wilschut J, de Haan A. Immunogenicity and protective capacity of a virosomal respiratory syncytial virus vaccine adjuvanted with monophosphoryl lipid a in mice. *PLoS ONE.* 2012;7(5):e36812.

186. Niewiesk S, Prince G. Diversifying animal models: the use of hispid cotton rats (Sigmodon hispidus) in infectious diseases. *Lab Anim.* 2002 Oct.;36(4):357–372.

187. Blanco JCG, Pletneva L, Boukhvalova M, Richardson JY, Harris KA, Prince GA. The Cotton Rat: An Underutilized Animal Model for Human Infectious Diseases Can Now Be Exploited Using Specific Reagents to Cytokines, Chemokines, and Interferons. *J Interferon Cytokine Res.* 2004 Jan.;24(1):21–28.

188. Wynn TA. IL-13 effector functions. *Annu Rev Immunol.* 2003;21:425–456.

189. Li-Weber M, Krammer PH. Regulation of IL4 gene expression by T cells and therapeutic perspectives. *Nat Rev Immunol.* 2003 Jul.;3(7):534–543.

190. Goswami R, Kaplan MH. A brief history of IL-9. *J Immunol.* 2011 Mar. 15;186(6):3283–3288.

191. Shen HH, Ochkur SI, McGarry MP, Crosby JR, Hines EM, Borchers MT, et al. A causative relationship exists between eosinophils and the development of allergic pulmonary pathologies in the mouse. *J Immunol.* 2003 Mar. 15;170(6):3296–3305.

192. Steenwinckel V, Louahed J, Orabona C, Huaux F, Warnier G, McKenzie A, et al. IL-13 mediates in vivo IL-9 activities on lung epithelial cells but not on hematopoietic cells. *J Immunol.* 2007;178(5):3244–3251.

193. Maes T, Joos GF, Brusselle GG. Targeting Interleukin-4 in Asthma: Lost in Translation? *American Journal of Respiratory Cell and Molecular Biology.* 2012 Apr. 26;

194. Gerard C, Gerard NP. C5A anaphylatoxin and its seven transmembrane-segment receptor. *Annu Rev Immunol.* 1994;12:775–808.

195. Höpken UE, Lu B, Gerard NP, Gerard C. Impaired inflammatory responses in the reverse arthus reaction through genetic deletion of the C5a receptor. *J Exp Med.* 1997 Aug. 29;186(5):749–756.

196. Humbles AA, Lu B, Nilsson CA, Lilly C, Israel E, Fujiwara Y, et al. A role for the C3a anaphylatoxin receptor in the effector phase of asthma. *Nature.* 2000 Aug. 31;406(6799):998–1001.

197. Kasturi SP, Skountzou I, Albrecht RA, Koutsonanos D, Hua T, Nakaya HI, et al. Programming

the magnitude and persistence of antibody responses with innate immunity. *Nature.* 2011 Feb. 24;470(7335):543–547.

198. Giudice EL, Campbell JD. Needle-free vaccine delivery. *Adv Drug Deliv Rev.* 2006 Apr. 20;58(1):68–89.

199. Hussell T, Humphreys IR. Nasal vaccination induces protective immunity without immunopathology. *Clin Exp Immunol.* 2002 Dec.;130(3):359–362.

200. Shafique M, Wilschut J, de Haan A. Induction of mucosal and systemic immunity against respiratory syncytial virus by inactivated virus supplemented with TLR9 and NOD2 ligands. *Vaccine.* 2012;30(3):597–606.

201. Childers N, Miller K, Tong G, Llarena J, Greenway T, Ulrich J, et al. Adjuvant activity of monophosphoryl lipid A for nasal and oral immunization with soluble or liposome-associated antigen. *Infect Immun.* 2000;68(10):5509–5516.

202. Baldridge JR, Yorgensen Y, Ward JR, Ulrich JT. Monophosphoryl lipid A enhances mucosal and systemic immunity to vaccine antigens following intranasal administration. *Vaccine.* 2000 May 8;18(22):2416–2425.

203. Pizza M, Giuliani MM, Fontana MR, Monaci E, Douce G, Dougan G, et al. Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants. *Vaccine.* 2001 Mar.;19(17-19):2534–2541.

204. Sasaki S, Hamajima K, Fukushima J, Ihata A, Ishii N, Gorai I, et al. Comparison of intranasal and intramuscular immunization against human immunodeficiency virus type 1 with a DNA-monophosphoryl lipid A adjuvant vaccine. *Infect Immun.* 1998 Feb.;66(2):823–826.

205. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. *Science.* 2007 Jun. 15;316(5831):1628–1632.

206. Parker D, Prince A. Innate immunity in the respiratory epithelium. *American Journal of Respiratory Cell and Molecular Biology.* 2011 Aug.;45(2):189–201.

207. Jia HP, Kline JN, Penisten A, Apicella MA, Gioannini TL, Weiss J, et al. Endotoxin responsiveness of human airway epithelia is limited by low expression of MD-2. *Am J Physiol Lung Cell Mol Physiol.* 2004 Aug.;287(2):L428–37.

208. Ainai A, Ichinohe T, Tamura S-I, Kurata T, Sata T, Tashiro M, et al. Zymosan enhances the mucosal adjuvant activity of poly(I:C) in a nasal influenza vaccine. *J Med Virol.* 2010 Mar.;82(3):476–484.

209. Cataldi A, Yevsa T, Vilte DA, Schulze K, Castro-Parodi M, Larzábal M, et al. Efficient immune responses against Intimin and EspB of enterohaemorragic Escherichia coli after intranasal vaccination using the TLR2/6 agonist MALP-2 as adjuvant. *Vaccine.* 2008 Oct. 16;26(44):5662–5667.

210. Johansen F-E, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor and IgA transport: new advances in environmental factors that stimulate pIgR expression and its role in mucosal immunity. *Mucosal Immunol.* 2011 Nov.;4(6):598–602.

211. Bruno MEC, Frantz AL, Rogier EW, Johansen F-E, Kaetzel CS. Regulation of the polymeric immunoglobulin receptor by the classical and alternative NF-κB pathways in intestinal epithelial cells. *Mucosal Immunol.* 2011 Mar. 30;4(4):468–478.

212. Renegar K, Small P, Boykins L, Wright P. Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract. *J Immunol.* 2004;173(3):1978–1986.

213. Renegar KB, Small PA. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. *J Virol.* 1991 Apr.;65(4):2146–2148.

214. Walsh EE, Falsey AR. Humoral and mucosal immunity in protection from natural respiratory syncytial virus infection in adults. *J Infect Dis.* 2004 Jul. 15;190(2):373–378.

215. Curtis SJ, Ottolini MG, Porter DD, Prince GA. Age-dependent replication of respiratory syncytial virus in the cotton rat. *Exp Biol Med (Maywood).* 2002 Oct.;227(9):799–802.

216. Boukhvalova MS, Yim KC, Kuhn KH, Hemming JP, Prince GA, Porter DD, et al. Age-related differences in pulmonary cytokine response to respiratory syncytial virus infection: modulation by anti-inflammatory and antiviral treatment. *J Infect Dis.* 2007 Feb. 15;195(4):511–518.

217. Aw D, Silva AB, Palmer DB. Immunosenescence: emerging challenges for an ageing population. *Immunology.* 2007 Apr.;120(4):435–446.

218. Grubeck-Loebenstein B, Bella Della S, Iorio AM, Michel J-P, Pawelec G, Solana R. Immunosenescence and vaccine failure in the elderly. *Aging Clin Exp Res.* 2009 Jun.;21(3):201–209.

219. Renshaw M, Rockwell J, Engleman C, Gewirtz A, Katz J, Sambhara S. Cutting edge: impaired Toll-like receptor expression and function in aging. *J Immunol.* 2002 Nov. 1;169(9):4697–4701.

220. Boehmer E, Goral J, Faunce D, Kovacs E. Age-dependent decrease in Toll-like receptor 4-mediated proinflammatory cytokine production and mitogen-activated protein kinase expression. *J Leukoc Biol.* 2004;75(2):342–349.

221. Boehmer E, Meehan M, Cutro B, Kovacs E. Aging negatively skews macrophage TLR2- and TLR4-mediated pro-inflammatory responses without affecting the IL-2-stimulated pathway. *Mech Ageing Dev.* 2005;126(12):1305–1313.

222. van Duin D, Mohanty S, Thomas V, Ginter S, Montgomery RR, Fikrig E, et al. Age-associated defect in human TLR-1/2 function. *J Immunol.* 2007;178(2):970–975.

223. Beyer WE, Palache AM, Sprenger MJ, Hendriksen E, Tukker JJ, Darioli R, et al. Effects of repeated annual influenza vaccination on vaccine sero-response in young and elderly adults. *Vaccine.* 1996 Oct.;14(14):1331–1339.

224. Belyakov IM, Ahlers JD. What role does the route of immunization play in the generation of protective immunity against mucosal pathogens? *J Immunol.* 2009 Dec. 1;183(11):6883–6892.

225. Fujihashi K, Kiyono H. Mucosal immunosenescence: new developments and vaccines to control infectious diseases. *Trends Immunol.* 2009;30(7):334–343.

226. Qureshi N, Takayama K, Ribi E. Purification and structural determination of nontoxic lipid A obtained from the lipopolysaccharide of Salmonella typhimurium. *J Biol Chem.* 1982 Oct. 10;257(19):11808–11815.

227. Polack FP. Atypical measles and enhanced respiratory syncytial virus disease (ERD) made simple. *Pediatr Res.* 2007 Jul. 1;62(1):111–115.

228. Steeghs L, Keestra AM, van Mourik A, Uronen-Hansson H, van der Ley P, Callard R, et al. Differential activation of human and mouse Toll-like receptor 4 by the adjuvant candidate LpxL1 of Neisseria meningitidis. *Infect Immun.* 2008 Aug.;76(8):3801–3807.

229. Walsh EE, Falsey AR. Age related differences in humoral immune response to respiratory syncytial virus infection in adults. *J Med Virol.* 2004 Jun.;73(2):295–299.

230. Dasari P, Zola H, Nicholson IC. Expression of Toll-like receptors by neonatal leukocytes. *Pediatr Allergy Immunol.* 2011 Mar.;22(2):221–228.

231. Nguyen M, Leuridan E, Zhang T, De Wit D, Willems F, Van Damme P, et al. Acquisition of adult-like TLR4 and TLR9 responses during the first year of life. *PLoS ONE.* 2010;5(4):e10407.

232. Murphy BR, Hall SL, Kulkarni AB, Crowe JE, Collins PL, Connors M, et al. An update on approaches to the development of respiratory syncytial virus (RSV) and parainfluenza virus type 3 (PIV3) vaccines. *Virus Res.* 1994 Apr.;32(1):13–36.

233. Englund J, Glezen WP, Piedra PA. Maternal immunization against viral disease. *Vaccine.* 1998 Aug.;16(14-15):1456–1463.

234. Waris ME, Tsou C, Erdman DD, Day DB, Anderson LJ. Priming with live respiratory syncytial virus (RSV) prevents the enhanced pulmonary inflammatory response seen after RSV challenge in BALB/c mice immunized with formalin-inactivated RSV. *J Virol.* 1997 Sep. 1;71(9):6935–6939.

235. Simoes EA, Tan DH, Ohlsson A, Sales V, Wang EE. Respiratory syncytial virus vaccine: a systematic overview with emphasis on respiratory syncytial virus subunit vaccines. *Vaccine.* 2001 Dec. 12;20(5-6):954–960.

236. Giannini SL, Hanon E, Moris P, van Mechelen M, Morel S, Dessy F, et al. Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. *Vaccine.* 2006 Aug. 14;24(33-34):5937–5949.